Proximate and ultimate aspects of androgen-mediated maternal effects in relation to sibling competition in birds

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The research reported in this thesis was carried out at the Department of Behavioural Biology, University of Groningen, The Netherlands, and in Ekkeroy, Norway in collaboration with NINA in Tromsø, Norway. All studies were approved by the Ethical Committee of the University of Groningen. The research was financially supported by an Ubo Emmius scholarship from the Faculty of Mathematics and Natural Sciences of the University of Groningen. The production of the thesis was financially supported by the Graduate School of Science (GSS) and the Faculty of Mathematics and Natural Sciences, University of Groningen.

Lay-out and figures:	Martina S. Müller
Cover illustration:	Martina S. Müller
Printed by:	Ipskamp Drukkers

RIJKSUNIVERSITEIT GRONINGEN

Proximate and ultimate aspects of androgen-mediated maternal effects in relation to sibling competition in birds

PROEFSCHRIFT

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, P. Luiten
in het openbaar te verdedigen op
maandag 1 juli 2013
om 9:00 uur

door

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geboren op 22 augustus 1980
te Basel, Zwitserland

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This thesis is dedicated to my teachers

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Chapter 1. Introduction and synthesis

Martina S. Müller

General background

Phenotypic plasticity is the primitive character state for most traits, as developmental, physiological and metabolic processes are inherently sensitive to mechanical and chemical forces in the environment (West-Eberhard 2003). Evolution may over time select for homeostatic mechanisms that stabilize the phenotype against such environment-induced variation (West-Eberhard 2003). Evolution may also exploit inherent plasticity if it enhances fitness, and over time select for a more fine-tuned plastic response (canalization of a reaction norm) that produces an adaptive range of phenotypic variation for a relevant range of environments (Pigliucci 2010). Such adaptive reaction norms often emerge due to specially evolved mechanisms that are regulated by variation in hormone secretion (Nijhout 2003). Hormones are chemical messengers that are secreted into the circulation and can therefore reach the whole organism, transducing environmental information, and synchronizing a wide array of adaptive behavioral, physiological and morphological phenotypic responses involving different tissues to environmental variation over the course of an organism's lifetime (Nelson 2005). Throughout the plant and animal kingdom, mothers also transmit these hormones to the offspring which strongly influences the developmental trajectories and abilities of offspring to survive and reproduce in the environment they are born into (e.g. von Engelhardt and Groothuis 2011, Mousseau and Dingle 1991). This process can enable adaptive inheritance of environmentally-induced variation in the parent's phenotype by the offspring, a process often referred to as a maternal effect (Groothuis et al. 2005, Marshall and Uller 2007).

While considerable evidence for the importance of maternal effects has accumulated over the past decades, it was largely ignored in the development of evolutionary theory until recently (Uller 2008). Increasingly, evolutionary biologists are expanding the formerly narrow gene-centered view of the Mendelian genetic framework of the Modern Synthesis to encompass also non-genetic forms of inheritance as they can strongly influence the course of evolution (Pigliucci 2010, Day and Boduriansky 2011). Maternal effects can retard adaptation by acting as a phenotypic buffer against selective forces, or by transmiting maladaptive phenotypic characteristics such as pathologies (Kirkpatrick and Lande 1989, Day and Boduriansky 2011). Maternal effects can also facilitate rapid adaptation to novel environments, for example via within-generation phenotypic responses to environmental change followed by non-genetic transmission of this response to subsequent generations, followed by eventual genetic accommodation of the most persistent adaptations (Badyaev and Uller 2009). The pathways of non-genetic inheritance are diverse, including transmission of behavioral variation through learning, transmission of aspects of the extra-organismal environment, molecular mechanisms such as variation in DNA methylation patterns and RNA, and transmission of nutrients, antibodies, and other resources, as well as diverse somatic factors including hormones (Ledon-Rettig et al 2012).

While hormone-mediated maternal effects have been studied in diverse taxa (e.g. plants: Donahue 2009; reptiles: Bowden et al. 2000; fish: Schaafsma and Groothuis 2012, mammals: Cottrel and Seckl 2009) many recent advances in the field have come from the wealth of observational and experimental studies conducted on birds (reviewed in von Engelhardt and Groothuis 2011). Two decades ago, avian egg yolks were reported to contain high concentrations of maternal testosterone, that vary widely within clutches, between clutches, between mothers and also between species (Schwabl 1993). At that time, birds had already proven to be excellent models in the field of behavioral ecology for studying many other types of maternal reproductive investment because all maternally-derived substances are transferred to the offspring in a large sealed egg developing outside of the mother's body, permitting easy access for measuring and manipulating different aspects of egg composition and onset or temperature of incubation. In addition, it is relatively easy to manipulate avian brood size, cross-foster offspring between broods, and quantify parental care behavior and the physiological and behavioral results of maternal effects in offspring (Gill 2007). Furthermore, studies of captive and free-living birds had been integral for advancing knowledge of comparative endocrinology,

and the diversity of their life histories and social systems provided ample opportunity for testing adaptive hypotheses (Adkins-Regan 2005).

While several steroid hormones besides testosterone are present in avian eggs, including progesterone, androstenedione, dihydrotestosterone, as well as low levels of estradiol and possibly corticosterone, until now, most of the work on maternal hormones in birds has focused on testosterone (von Engelhardt and Groothuis 2011). Extensive experimental evidence has accumulated for diverse effects of elevated maternal testosterone on offspring development during the pre-natal period, the post-natal period and even into adulthood (von Engelhardt and Groothuis et al. 2011). These studies elevated the testosterone content of egg yolks to the upper physiological range usually by injecting freshly-laid eggs into the yolk sac, with a dose of a known quantity of a specific androgen dissolved in oil. The yolk sac provides the embryo with continuous nourishment and also other maternally-derived substances including testosterone throughout pre-natal development and in precocial species even for a few days after hatching (Gill 2007). Pre-natal exposure to elevated yolk testosterone often produces chicks with a more competitive phenotype by accelerating growth, increasing begging and aggressive behavior, and enhancing survival, although it carries some costs of elevated metabolic rate and compromised immune function (reviewed in von Engelhardt and Groothuis 2011).

The larger focus on yolk testosterone compared to other yolk hormones, began partly due to the emphasis on testosterone in the field of behavioral ecology because of its importance in regulating numerous physiological and behavioral aspects of reproduction. Interest in testosterone in eggs continued, as it has been reliably present in relatively high concentrations in egg yolks of all species in which it has been measured. Furthermore it has been found to vary systematically within clutches over the laying sequence in different patterns (Schwabl 1993, reviewed in Groothuis at al 2005, Gil 2008), between clutches in accordance with variation in the environment, food availability, mate quality or the mother's condition (reviewed in von Engelhardt and Groothuis 2011) and between species in ways predicted by their life histories (Gorman and Williams 2005, Gil et al. 2007, Schwabl et al. 2007) which generated several interesting hypotheses about the evolutionary origins of the observed patterns.

For example, the frequent increasing pattern of yolk testosterone concentrations over the laying sequence in many species has been suggested to represent adaptive maternal compensation for the competitive disadvantage experienced by the latest-hatching chicks in a brood (Groothuis et al. 2005), although why mothers would create hatching asynchrony and then counteract its effects via testosterone allocation remains unresolved. One solution to this problem is that effects of within-clutch patterns of maternal testosterone work in concert with adaptive context-dependent effects of hatching asynchrony. Hatching asynchrony facilitates brood reduction in poor years via starvation of late-hatching chicks, but permits their survival when resources are abundant. An increasing pattern of maternal testosterone over the laying sequence may compound the negative effects of hatching late in undermining survival of late-hatching chicks poor years, but in good years aid late-hatching chicks in overcoming their competitive disadvantage and boost their survival. Until now, such context-dependent effects of maternal testosterone have not yet been directly tested.

A decreasing pattern of yolk testosterone over the laying sequence has been suggested to be an adaptive mechanism to support brood reduction in siblicidal species (Schwabl et al. 1997). Until now substantial correlative work (e.g. Schwabl et al. 1997, Drummond et al. 2008, Ferree et al. 2004, Nuñez de la Mora et al. 1996, Ramos-Fernandez et al. 2000, Tarlow et al. 2001) has addressed the potential function of pre-and post-natal testosterone for siblicidal aggression but it has never been tested experimentally.

But whether maternal testosterone in the eggs correlates with testosterone in the mother's circulation is still not known although this has important ramifications for their abilities to evolve independently. Furthermore, which selection pressures have shaped maternal testosterone in eggs vs. the mother's circulation have been investigated separately, but not yet within the same comparative study.

This thesis evaluates several of these proximate- and ultimate-level hypotheses about hormone-mediated maternal effects. I have addressed them via observational and experimental

studies on individuals from the same species, and by investigating the origins of between-species variation in hormone-mediated maternal effects using several phylogenetically-controlled comparative analyses.

Hormone-mediated maternal effects: adaptive mediators of sibling competition and brood reduction?

According to the parental favoritism hypothesis (Mock and Parker 1997), mothers produce an optimistic number of young (in anticipation that breeding conditions may be very good) but at the same time create competitive hierarchies among offspring so that in case resources become scarce, more resources are channeled to the more competitive siblings so that the less competitive siblings starve to death quickly, adjusting the brood size to a size that parents can afford to rear given the current resource availability. This resolves the evolutionary trade-off between quantity and quality that arises due to resource limitations because it allows parents to maximize the number of young they can rear in current conditions, but not compromise the quality of the young.

Mothers have a diverse toolkit by which they modify competitive abilities in offspring. In birds, one of the primary pathways is by commencing incubation before clutch completion, which initiates development of the early-laid (core) eggs before that of late-laid (marginal) eggs, resulting in asynchronous hatching. The fact that hatching asynchrony might be an adaptation to facultatively adjust brood size was first recognized by David Lack (1947, 1954, 1968) and articulated in what is today referred to as the "resource-tracking hypothesis". Hatching asynchrony imposes a severe competitive handicap on marginal chicks because by the time they emerge from the egg, core chicks have already grown and developed to a degree commensurate with the hatching spread and their growth rate. The inferior competitive ability of marginal young makes them much more susceptible to dying when food conditions deteriorate (Mock 1984, Magrath 1990), buffering the core part of the brood from starvation and adaptively reducing brood size.

The discovery that in some species yolk androgen concentrations increase in consecutively-laid eggs and in other species they decrease, inspired the idea that an increasing pattern of yolk androgens over the laying sequence (i.e. relatively more androgens for marginal chicks than core chicks, Schwabl 1993) indicates that mothers aim for their entire brood to survive and a decreasing pattern (i.e. relatively more androgens for core chicks than marginal chicks, Schwabl et al. 1997) indicates that mothers aim to reduce their broods (hatching asynchrony adjustment hypothesis - HAAH, Groothuis et al. 2005). Over time, some problems with this particular formulation have emerged that we elucidate in **Chapter 3**. First of all, the HAAH assumes that hatching asynchrony is a problem for the brood survival species because it sabotages the ability of parents to rear a full brood of high quality chicks, and this problem needs to be solved by bestowing marginal chicks with extra testosterone. This goes against the resource-tracking and parental favouritism hypotheses, which posit that hatching asynchrony is adaptive. Past studies have suggested that hatching asynchrony may have evolved as a costly side-effect of adaptive early onset of incubation to protect eggs from predators or adverse weather conditions (reviewed in Magrath 1990) and therefore need compensation, but until now much more evidence supports the idea of hatching asynchrony as an adaptation (Amundsen and Slagsvold 1991) rather than as an epiphenomenon (Magrath 1990).

Secondly, assuming that hatching asychrony creates competitive asymmetries to adaptively promote partial reduction of broods in unexpectedly poor conditions but permit survival of the full brood in unexpectedly favorable conditions (as stated in the resource-tracking and parental favoritism hypotheses), then the HAAH's classification of species as purely brood survival or brood reduction species creates a false dichotomy. Most species providing post-natal parental care (and producing more than one egg in a clutch) are unlikely to be obligate brood survival species because they create some degree of hatching asynchrony and rear fewer offspring when resources become scarce. Obligate brood reducers, which produce marginal eggs purely for insurance purposes (in case the core offspring fail), are also very rare

(Anderson 1990). This means that most species are facultative brood reducers in which all young are reared under good conditions and marginal chicks are eliminated in poor conditions. This had led to inaccurate predictions for yolk androgen allocation patterns based on the HAAH. For example, after the prominent finding that yolk androgens show a decreasing pattern over the laying sequence in the siblicidal cattle egret (Schwabl 1997), and given that a siblicide is a behavioural mechanism for brood reduction, siblicidal species were generally expected to show a decreasing pattern of androgens over the laying sequence. This has since been found not to be true, as most siblicidal species show an increasing pattern (e.g. black-legged kittiwake, **Chapter 4**). Indeed, most siblicidal species are facultative brood reducers, in which the core siblings kill their younger siblings only when food becomes scarce enough that the risk to their own survival poses a larger fitness cost than the cost to their inclusive fitness they incur by killing their sibling (Mock and Parker 1997). The reason why an obligate brood reduction strategy exists, is that certain species run a high risk of losing high value core chicks due to poor hatching success, and therefore produce marginal eggs as an insurance policy (Anderson 1990). Species producing marginal chicks purely for insurance purposes are not able to rear the whole brood (otherwise they would occasionally aim for a brood survival strategy) so they create especially large competitive handicaps for marginal chicks so that they are easily eliminated in case all core chicks survive (Anderson 1990).

This motivated a new hypothesis that I propose and test in **Chapter 3**, stating that patterns of within-clutch yolk testosterone concentrations should vary with the degree to which mothers produce marginal chicks for insurance (as replacement units in case a high value core chicks dies) vs. for reaping a reproductive "bonus" in unexpectedly good years when parents can afford to rear both the core and marginal parts of the brood. I expected (1) that species with the insurance strategy, associated with large hatching asynchrony or fast growth rates (both of which produce large size asymmetries between core and marginal young) should produce a flat or decreasing pattern of yolk androgens over the laying sequence. I expected this because in such species, marginal chicks never survive alongside core chicks; therefore compensating their competitive handicap would never help them, it would only make efforts of core offspring in outcompeting marginal siblings more costly. As marginal chicks in such species only survive when the core chicks die, marginal chicks would not benefit from a competitive boost coming from compensatory maternal testosterone. I also expected (2) that species with the strategy of producing marginal young as a gamble, create smaller competitive handicaps (narrower hatching asynchrony or slower growth rates) so that marginal chicks are able to overcome their disadvantage when resources are plentiful and also produce an increasing pattern of yolk androgens over the laying sequence which might boost the competitive ability of these marginal chicks. In reality, most species fall somewhere between the two extremes and receive insurance value from marginal young in poor years and a reproductive bonus when the full brood survives in good years although the relative importance of insurance value and "bonus" value varies widely between species.

Consistent with my predictions, in an interspecies analysis, I found a robust negative correlation between the size asymmetries between core and marginal chicks and the amount of compensatory testosterone mothers give to marginal chicks (i.e. the relative amount given to marginal chicks compared to core chicks) showing that mothers only compensate marginal eggs with extra testosterone if they are similar in size to core chicks. I also found that hatching asynchrony and growth rate (which both affect the competitive disadvantage of marginal chicks), both correlated negatively to compensatory testosterone in marginal chicks, consistent with the previous finding and with our expectations. The relationships we found indicate that yolk testosterone compensation has evolved in concert with these other mechanisms that contribute to size asymmetries within the brood. Therefore, consistent with the original formulation of the hatching asynchrony adjustment hypothesis, patterns of yolk androgens over the laying sequence appear to have evolved to "adjust hatching asynchrony" and an increasing pattern is expected to promote survival of the whole brood while a decreasing or flat pattern is expected to promote partial reduction of the brood. But I have placed these ideas into a novel evolutionary framework that avoids the strict dichotomy between brood survival and brood

reduction strategy, assumes size asymmetries within broods are an adaptation (which is much more plausible than the former interpretation as a maladaptive epiphenomenon), and acknowledges for the first time that variation in size asymmetries arises not only due to variation in hatching spreads but also in varying growth rates.

An unresolved problem until now not addressed in the literature concerns the fact that while an increasing pattern of yolk androgens can promote full survival of the brood during good years, in poor years the positive effects of high maternal androgen exposure in marginal chicks could work against the negative effects of hatching asynchrony in eliminating marginal young in poor years, making adaptive brood reduction less efficient. But this problem is resolved if yolk testosterone has beneficial effects only in good breeding conditions and imposes more costs than benefits in poor breeding conditions. For example, the amount of food delivered to broods of growing nestlings might modulate whether relatively higher testosterone concentrations in the yolks of eggs that produce marginal chicks, promote brood survival or brood reduction: testosterone can increase competitive ability to obtain food so the accompanying elevated energy expenditure might be more than compensated by higher food returns when food is abundant but might not be fully compensated by higher food returns when food is scarce.

To test this hypothesis, we performed an experiment described in **Chapter 6** in rock pigeons (the wild ancestor of the domesticated pigeon), which produce a modal clutch size of two eggs in which maternal testosterone concentrations in the yolk are consistently much higher in the second egg than in the first egg. In a large colony of captive rock pigeons kept under semi-natural conditions, we collected freshly-laid first eggs (which contain low concentrations of testosterone) and injected half of them with an amount of testosterone solution that raises the hormone concentration to that of the second-laid egg, and injected the other half of the eggs with vehicle only. Hatchlings from opposite treatments were paired in a manner that matched their ages, sex and sizes, and were cross-fostered into nests of parents that were kept under restricted food conditions or into nests of parents that were kept under *ad libitum* food conditions.

Under *ad libitum* food conditions, chicks from testosterone-injected eggs (T-chicks) grew faster than did their control foster siblings, whereas under restricted food conditions, T-chicks had lower survival than their control foster siblings. As embryonic testosterone exposure can boost energetically-costly competitive behaviour for food (e.g. Eising and Groothuis 2003, Müller et al. 2012), which would be profitable when food is abundant (resulting in faster growth in T-chicks than in control chicks, **Chapter 6**), but potentially result in a detrimental, negative energy balance when food returns are insufficient to compensate the extra energy expenditure (resulting in higher mortality in T-chicks than in control chicks, **Chapter 6**), it is very possible that energy limitation mediated the observed differences among T treatment groups and food conditions.

We also found that under *ad libitum* food conditions, chicks showed a higher immune response than under food restriction, suggesting that insufficient energy and/or nutritional inputs reduce immunity. If this is the case, then the weaker immune response found in T-chicks compared to control-chicks under *ad libitum* food conditions might occur because exposure to elevated maternal testosterone diverts limited resources away from maintaining immune function to support energetically-costly competitive behaviour and higher basal metabolism (Tobler et al. 2007). In designing our experiment, we assumed that even when parents have access to unlimited food, the food delivered to offspring remains below a nestling's *ad libitum* level so chicks benefit from higher food returns coming from enhanced competitive ability induced by higher maternal T exposure (confirmed by faster growth in T-chicks). The fact that under good food conditions T-chicks had higher growth and lower immunity than control chicks did, suggests that even if food returns are high, some level of resource limitation remains, either due to limited food deliveries or physiological constraints in assimilating energy. What remains unresolved is a physiological explanation for why T-chicks had lower survival compared to control chicks in poor conditions as we did not find differences in growth and immunity for T-

and control-chicks under poor conditions, which suggests that a third factor that we did not measure was the direct cause of higher death rates in T-chicks.

Our findings that exposure to higher maternal testosterone enhances offspring quality in good conditions but undermines their survival in poor conditions supports our hypothesis and resolves the above described paradox. It also provides the first solid evidence for a key assumption that I made in **Chapter 3** to reconcile the findings of our comparative study with the parental favouritism hypothesis: that effects of exposure to elevated maternal testosterone (in marginal chicks) are beneficial in good conditions and detrimental in poor conditions.

In light of my findings in **Chapter 6**, it is possible that the inconsistent results coming from different studies testing effects of exposure to elevated maternal androgens occurred because differences in experimental conditions modulated the effects of yolk androgen exposure (assuming that the methodology of the egg injection approach is sound). The idea of adaptive context-dependent hormone-mediated maternal effects emphasizes the importance of publishing also the negative findings from yolk androgen experiments and the importance of describing experimental protocols and conditions in detail.

After resolving the paradox of an increasing pattern of yolk androgens over the laying sequence in species with frequent starvation-mediated brood reduction, an additional unresolved paradox remains for siblicidal species. Maternal androgen exposure, which has been shown to increase territorial aggression towards non-siblings in black-headed gulls (Müller et al. 2009), but also increases the ability to compete against siblings for food by means of begging (parental food solicition displays) (reviewed in von Engelhardt and Groothuis 2011), has been suggested to have a function in enhancing overt aggression directed at nest mates in siblicidal species, instrumental for killing or removing the marginal chick by the core chick (Schwabl et al. 1997). The decreasing pattern of yolk androgens over the laying sequence reported for the siblicidal cattle egret (Schwabl et al. 1997) was interpreted as a maternal strategy to support the socially dominant first-hatching chick in killing its subordinate younger sibling. But since then several studies have reported flat or increasing patterns in siblicidal species (reviewed in von Engelhardt and Groothuis 2011). In **Chapter 4,** I show that in the siblicidal black-legged kittiwake, testosterone and androstendione concentrations are much higher in the second-laid marginal egg than in the first-laid core egg which goes in the opposite direction of the direction of the pattern found in cattle egrets and casts doubt on the idea that yolk testosterone exposure would support efficient siblicide-mediated elimination of marginal chicks by core chicks. I then performed an experiment to test whether elevated maternal androgen exposure increases siblicidal aggression and begging. I collected freshly-laid first eggs (which have a low yolk androgen level) from a kittiwake colony, and injected half of them with androgens (to increase the concentration to that of a second-laid marginal egg) and the other half with vehicle and then paired chicks hatching from androgen eggs with chicks coming from control eggs, matching foster siblings in age and size. To my surprise, exposure to elevated androgens, as occurs in marginal chicks, increased sibling aggression and dominance status although this potential for aggression is clearly suppressed in a natural setting because chicks from second eggs are subdominant due to the substantial size disadvantage (Braun and Hunt 1983, see **Chapter 5**). This presents the following paradox: nest mates from siblicidal species such as kittiwakes, create strong dominance hierarchies in which the core chick invariably has dominant status and pushes, pecks and at times even kills its competitively inferior marginal sibling that actually hatches from eggs with higher testosterone levels. The marginal sibling, however, never expresses any aggressive behaviour in return and usually tucks its bill under the body exposing the back of the neck in a submissive posture that appeases the core sibling. If the aggression-enhancing effects of higher androgen exposure in marginal chicks are never expressed due to their subordinate status, why then do mothers put more androgens into those eggs?

One possibility is that compensatory maternal androgens exposure for marginal kittiwake chicks evolved to enhance begging and ability to compete for food. However, in my experiment I found that elevated yolk androgens did not enhance begging which may be due to the fact that in my experimental setup chicks were individually hand-fed and did not have to compete for food. But when placed in a situation in which they did have to compete for food,

chicks exposed to elevated yolk androgen exposure performed better when competing for food deliveries. Another possibility is that the strong effects of androgen exposure in increasing siblicidal aggression do have fitness benefits for marginal chicks, but just not in their natal nest. Kittiwakes breed in dense colonies situated on cliffs and core chicks eliminate siblings via an onslaught of pecking that ultimately drives the marginal chicks over the edge of the nest cup. Very often, ejected chicks fall into other nests below, and 8% of the time become adopted in new families (Roberts and Hatch 1994, Pierotti and Murphy 1987, Helfenstein et al. 2004) as parents do not recognize young until they are already several weeks old (Cullen 1957, Roberts and Hatch 1994). If ejected marginal chicks fall into nests containing other chicks of similar or slightly smaller size, pre-natal exposure to elevated maternal androgens would help them compete their way into dominant status, which would allow them to remain in that nest. The "adoption facilitation hypothesis" also falls in line with past findings that mothers tend to increase clutch average yolk androgen concentrations under poor food conditions (Gasparini et al. 2007, Verboven et al. 2003) and at higher breeding density (**Chapter 2**) in species that show adoption (reviewed in **Chapter 2**) which should increase the success of their marginal chicks in foster nests and increase the effectiveness of siblicidal core chicks in eliminating marginal chicks as well as potential intruding vagrant chicks.

Testosterone was first identified as a potent agent in enhancing aggression not via pre-natal organizational influences of maternally-derived testosterone on the development of aggressive behavior, but via the effects of circulating testosterone on short-term activational facilitation of behaviour in adults (Nelson 2005). Since then, circulating testosterone has also been shown to increase territorial aggression in very young black-headed gull chicks (Groothuis and Meeuwissen 1992, Ros et al. 2002, Groothuis and Ros 2005). But whether testosterone plays a role in the extremely violent attacks that occur among nest mates in siblicidal species has not yet been determined, although interest in the question has persisted for several decades (Nuñez de la Mora et al. 1996, Ramos-Fernandez et al. 2000, Tarlow et al. 2001). Ferree et al. (2004) showed that obligately siblicidal booby chicks dramatically increase testosterone concentrations during attacks on marginal siblings, suggesting that testosterone is important in facilitating sibling aggression. In a field experiment, I tested, for the first time, whether testosterone increases siblicidal aggression in black-legged kittiwake nestlings (**Chapter 5**). I implanted chicks with either a hormone-releasing capsule that contained testosterone or an empty capsule (control) and paired testosterone chicks with control chicks that were similar in age and size. I found that compared to control chicks, testosterone chicks were more aggressive toward their companion chick as well as toward a simulated predator and they also begged more to parents, indicating that circulating testosterone is a potent agent for enhancing competitive ability and aggression. I also found that the larger chicks within the experimental pairs were more aggressive chicks, which is consistent with the fact that the older, larger siblings in kittiwake nests are socially dominant and perform most of the aggression. This is the first experimental evidence that early postnatal T elevation facilitates chick aggression in a siblicidal context.

I also show that chicks with low residual body mass were more aggressive than were chicks with high residual body mass, as expected in a facultatively siblicidal species such as the kittiwake, in which siblicide only occurs under poor food conditions. Residual body mass is a proxy for an animal's energy reserves, reflecting its body mass after correcting for its structural body size. Facultatively siblicidal (core) chicks exhibit high levels of aggression and commit siblicide only when they are threatened with starvation and need to eliminate siblings so that they receive enough food to survive. Previous work in facultatively siblicidal blue-footed boobies, demonstrated that aggression increases dramatically only when body mass of a core chick falls below a certain threshold (Drummond and Garcia Chavelas 1989), suggesting the actions of a hormonal signal that reflects body condition. Corticosterone, the main stress hormone in birds, becomes elevated under food stress in chicks (e.g. Kitaysky et al. 1999). Corticosterone also increases aggression in kittiwake chicks (Kitaysky et al. 2003, M. S. Müller, unpublished data). Then why do chicks increase testosterone during siblicide and why did I find that testosterone increases siblicidal aggression? Testosterone, usually increases rapidly in reaction to a social challenge (Wingfield et al. 1990). If elevated corticosterone during poor food

conditions makes core chicks intolerant of their nestmates, they might suddenly perceive the presence of their nest mate as a social challenge that triggers the elevation of testosterone which facilitates aggression. The experiments that showed that corticosterone elevation increases aggression in kittiwakes, are not inconsistent with this hypothesis, as higher corticosterone could have made chicks perceive their nest mates as an intruder and such a stimulus may have caused endogenous testosterone up-regulation to facilitate the observed increased aggression. Future studies should examine whether testosterone increases siblicidal aggression in facultatively siblicidal species even when food conditions are favourable and circulating corticosterone concentrations are low and whether facultatively siblicidal chicks up-regulate testosterone in the presence of a nest mate only if corticosterone levels are high. Because I performed the field experiment during a time when the colony was struggling with low food availability, the chicks in my study most likely had high corticosterone levels, although sufficient variation remained in body condition that I was able to detect a relationship between residual body mass and aggressive behaviour. The planned experiment to test synergic effects of corticosterone and testosterone on kittiwake chick's aggression failed due to logistical difficulties in obtaining a sufficient number of viable eggs.

Maternal yolk testosterone and maternal plasma testosterone

The field of hormone-mediated maternal effects has flourished in behavioral ecology, yet many crucial gaps remain in our knowledge about proximate causes, for example whether maternal hormones that enter the yolk and influence the development of the offspring correlate with the maternal hormones that enter the mother's bloodstream and influence her behaviour and physiology. This question has important ultimate-level ramifications as it determines the degree to which maternal yolk hormones and maternal plasma hormones can evolve independently. Because the bulk of the maternal testosterone produced during the egg-laying period comes from the highly steroidogenic follicular tissues surrounding the growing yolks in the ovary, it is likely that species with a higher rate of testosterone synthesis have higher testosterone concentrations both in the yolks and in the mother's circulation, resulting in a positive correlation between yolk testosterone and maternal plasma testosterone. If selection is acting on testosterone production to optimize transfer to the mother's blood, then the testosterone concentrations in the egg represent a physiological epiphenomenon or exaptation (or vice versa). Several previous studies have attempted to test whether this is the case by performing correlations of maternal plasma testosterone and yolk testosterone within the same species, but have found very inconsistent patterns, most likely due to the rapid fluctuations in maternal plasma testosterone during the egg-laying period (reviewed in Groothuis and Schwabl 2008). But in my interspecies phylogenetic analyses described in **Chapter 2**, I found a strong positive correlation between species averages of maternal plasma testosterone and yolk testosterone concentrations.

In this analysis, including clutch size (i.e. the number of steroidogenic follicles) made the positive correlation between female plasma testosterone and yolk testosterone much stronger. Clutch size itself also correlated directly with female plasma testosterone, indicating that the amount of maternal testosterone that is secreted into the mother's circulation depends also on the number of hormone-producing follicles. These findings suggest that testosterone produced in the ovaries that enters the yolk and the mother's circulation is not regulated independently in birds. But the spread of the values along the positive correlation between female plasma testosterone and yolk testosterone indicates substantial interspecies variation in the rate of testosterone secretion by a single follicle.

Maternally derived testosterone concentration in eggs: a product of direct selection or an epiphenomenon?

Given that the activational effects of circulating maternal testosterone in the mother's blood and the organizational effects of exposure to maternal testosterone throughout embryonic development in offspring differ so much, as do their ecologies and environmental challenges, the pressures exerted by natural selection on maternal plasma testosterone and yolk testosterone are likely to differ substantially in both direction and intensity. Yet in **Chapter 2** I show that a positive correlation between them exists, suggesting that either a conflict in selection pressures between them was never strong enough for novel mechanisms allowing complete independent regulation to evolve, such phenotypes never arose for selection to act on, sensitivity to maternal testosterone rather than the testosterone concentrations themselves has changed in mothers or embryos, or the unlikely possibility that variation in maternal plasma testosterone and yolk testosterone are the product of separate but identical direct selective regimes.

Although the more likely scenario is that concentrations of maternal plasma testosterone and yolk testosterone are limited in the degree to which they are independently regulated, in **Chapter 2** I tested whether they correlate with the same life history traits and therefore might have been shaped by identical selection pressures. In **Chapter 2** I provide evidence from comparative analyses of life history traits, that over evolutionary time, maternal testosterone production has adapted to accommodate functions in the offspring in concert with the emergence of different life history traits. I found associations between coloniality and incubation period with yolk testosterone but not with maternal plasma testosterone, suggesting that the evolution of coloniality selected ovaries to produce a high testosterone level and the evolution of short incubation periods selected for ovaries to produce a low testosterone level, both to influence the offspring. Short incubation periods were also associated with high plasma testosterone in males but not in females. I interpreted the effect in males as reflecting a correlation with a third variable of a high predation rate as high predation rate has been shown to be a strong selective force in shortening incubation periods in diverse avian species. I suggest that when predation risk is high (causing incubation periods to be low), males benefit from having high testosterone concentrations because it causes them to have longer bouts of being on or off the nest and the fewer trips to and from the nest reduce the chances of attracting predators. On the other hand, exposure to yolk testosterone enhances conspicuous begging displays in chicks, which would attract predators to the nest, explaining why yolks appear to have been selected to contain low testosterone concentrations in predator-rich environments. The idea that high predation rates might be the cause of the opposite relationships between testosterone and incubation period (positive for yolk testosterone and negative for male plasma testosterone, both to prevent nest predation) and given the fact that high plasma testosterone influences incubation behavior in a similar way in females as in males, the weak negative relationship that we found between female plasma testosterone and incubation period may reflect constraints of opposing pressures on maternal testosterone and yolk testosterone. We found a similar pattern (in which the relationship of female plasma testosterone and a life history trait falls between the relationship of the same life history trait with yolk testosterone and male plasma testosterone) also for coloniality and mating system.

Evolutionary processes underlying species differences in maternal T production

In the last decade, hormone concentrations have been the subject of several comparative studies, and variation among species values are often implied to reflect evolutionary change in the underlying genes, but a species' hormone concentration might not necessarily be genetically restricted to a specific value. Both yolk testosterone and plasma testosterone within species have been shown to vary in predictable ways along many environmental gradients, mediating several plastic behavioral and physiological aspects of the phenotype in ways that have been suggested to enhance fitness (reviewed in von Engelhardt and Groothuis 2011), which would make these reaction norms adaptive properties of the genotypes. In fact, the binary species values for the life history traits examined in this study may represent polarized values along the

same social/environmental gradients (e.g. colonial vs. not colonial) and the associated contrasts in testosterone levels might simply reflect opposite ends of a continuum along these gradients. This idea that the variation in species' testosterone levels reflects different locations along the same reaction norms has several implications. First of all, the unexplained variation in the correlation between yolk testosterone and plasma testosterone would indicate that yolk testosterone and plasma testosterone do not have perfectly parallel reaction norms. This could come about by a variety of mechanisms that might vary with increasing ovarian testosterone production such as testosterone clearing rates from the mother's plasma or testosterone conversion activity of enzymes present in the follicle or oocyte.

Furthermore, if interspecies testosterone variation reflects different locations along the same reaction norms, then assuming that testosterone production retained its plasticity, testosterone levels would converge if the social/environmental circumstances converged and differences in testosterone-mediated behavior and physiology in these species would not be genetically-fixed species traits at all. But if natural selection keeps operating in the same environment over evolutionary time, the phenotype may become genetically fixed (assimilated) and lose its plasticity, for example due to drift or selection arising from costs of maintaining plasticity (Schmalhausen 1949, West-Eberhard 2003, Pigliucci et al. 2006).

Yolk testosterone deposition has been shown to respond to artificial selection experiments in Japanese quail (Okuliarova et al. 2011) suggesting the presence of some underlying genetic architecture. Further evidence for some genetic basis to maternal testosterone production comes from several studies which have reported significant individual repeatability in yolk hormone deposition in songbird species (zebra finches: Bolund et al. 2008, Sandell et al. 2007; starlings: Eising et al. 2008; house martins: Gil et al. 2006; barn swallow: Gil et al. 2006; flycatcher: Tobler et al. 2007; tits: Tschirren et al. 2009), and yolk testosterone deposition of daughters significantly correlated with that of mothers in a study on tits (Tschirren et al. 2009) but not in a study on canaries (Müller et al. 2012). However, these estimates may be inflated by maternal effects in the egg such as maternal testosterone (Tschirren et al. 2009) although until now, no evidence exists that yolk testosterone exposure influences yolk testosterone deposition in females or plasma testosterone in adulthood (Müller et al. 2011). The heritability of plasma testosterone levels, on the other hand, has hardly been investigated in birds. Clearly many questions remain about the degree to which variation in species means of plasma testosterone or yolk testosterone have a genetic basis.

Conclusions and future directions

Over the past two decades the importance of maternal effects for phenotypic plasticity, heritability and evolution has become firmly established. The main aim of this thesis was to elaborate and test ultimate and proximate-level hypothesis about hormone-mediated maternal effects in birds, particularly in the context of sibling rivalry. Under favorable breeding conditions (i.e. when food is plentiful), embryonic exposure to elevated maternal androgens enhances competitive behaviour (**Chapter 4**) and growth (**Chapter 6**) in nestlings. In birds, yolk androgen concentrations vary systematically over the laying sequence in patterns that vary widely between species. As later-hatching (marginal) chicks within a brood often are exposed to higher maternal testosterone concentrations than early-hatching (core) chicks (an increasing pattern of testosterone over the laying sequence), these effects can potentially boost the survival of the developmentally disadvantaged marginal chicks. But exposure to elevated maternal testosterone also imposes costs such as reduced immune function (**Chapter 6**), and under poor conditions can increase mortality of marginal chicks (brood reduction, **Chapter 6**).

Mothers overproduce young and marginal chicks can provide 1) a reproductive bonus in breeding conditions that permit survival of the whole brood and/or 2) they can serve as replacement units in case a core chick dies and these reproductive strategies correlate with patterns of maternal testosterone over the laying sequence. The former strategy is associated with small competitive size asymmetries due to narrow hatching spreads or slow growth rates and an increasing pattern of maternal testosterone over the laying sequence (**Chapter 3**). The

latter strategy is associated with large competitive size asymmetries within broods due to broad hatching spreads or fast growth rates and a decreasing pattern of maternal testosterone over the laying sequence (**Chapter 3**).

But the concentrations of ovarian testosterone that enter egg yolks correlate with the testosterone released in the mother's own plasma (**Chapter 2**) which may impose constraints on selection in optimizing testosterone exposure both in offspring and the mother. This raises the question whether the mothers' ovaries have evolved to produce testosterone in a manner that accommodates the offspring or the mother. It also raises the possibility that examining patterns in maternal testosterone in yolks separately from patterns in maternal testosterone in mothers paints an incomplete and potentially misleading picture. We found evidence that selection appears to have generated adaptive variation in both yolk testosterone and male plasma testosterone but were unable to find evidence for adaptive variation in female plasma testosterone (**Chapter 2**).

The work described in this thesis has generated several other interesting questions that now need to be experimentally tested. For example, does prenatal exposure to elevated maternal androgens increase survival of chicks by increasing their chances of becoming adopted in nearby nests (**Chapter 4**)? Does corticosterone make "core" chicks intolerant of their "marginal" nest mates and turn their younger siblings into a social stimulus that triggers testosterone-mediated siblicidal aggression (**Chapter 5**)? How do the organizational effects of exposure to pre-natal (maternal) and the activational effects of post-natal (endogenous) testosterone interact?

Also, further studies are necessary to understand how mothers change yolk androgen allocation in response to prevailing food conditions during egg formation: how does this cause patterns over the laying sequence and clutch average concentrations to vary? As the hypothesized adaptive functions of hormone-mediated maternal effects are to act as a "weather forecast" to provide offspring with information about prevailing environmental conditions, if the environment changes unpredictably and the forecast is wrong, mechanisms should evolve that permit the current environment to modulate the influence of the maternal effect on the offspring's development, essentially creating an environment x environment interaction. We found the first evidence of this (**Chapter 6**) but are left with the unresolved question: what was the cause of the higher mortality observed in chicks exposed to higher yolk testosterone that were reared in poor food conditions?

Chapter 2 suggests that maternal plasma testosterone and yolk testosterone are not completely independently regulated, and that selection acting on yolk testosterone might constrain maternal plasma concentrations, but more work needs to be done in 1) identifying the proximate mechanisms underlying hormone transfer between the mother's ovary and her circulation as well as the developing yolks and 2) in studying the effect of physiologically relevant concentrations of testosterone on female behaviour and physiology during the egg-laying period to determine whether high or low testosterone in females is selectively neutral, beneficial or imposes fitness costs.

One important area of inquiry, which I have not addressed in this thesis, concerns the fact that patterns of yolk androgen concentrations over the laying sequence vary substantially within species and even between clutches from the same females. Figure 1 shows the variation in patterns within several taxonomically diverse avian species and reveals that species averages indicating that a flat pattern of yolk androgens over the laying sequence (slope = 0) actually contains several clutches showing increasing and decreasing patterns. Even species averages indicating a steep increasing pattern of yolk androgens can also contain individuals producing clutches with decreasing patterns (e.g. *Larus ridibundus*). This large variation raises several interesting questions: for example, to what degree do species differences in yolk androgens reflect evolved differences between species and to what degree do they simply reflect differences in environmental circumstances between the species? The fact that the pattern over the laying sequence as well as average clutch levels still differs substantially between different species kept in similar conditions in captivity (e.g. *Serinus canaria, Taenopygia guttata*, and *Columba livia*, Figure 1,2), and the fact that there are no obvious differences in patterns between captive and wild populations (e.g. Griffith et al. 2011, Gil et al. 1999, 2004, Rutstein et al. 2004,

Gilbert et al. 2005, 2007) suggests that consistent differences between species in these patterns exist and they do not just reflect environmental variation.

Future comparative studies should quantify the degree of variation in clutch averages of yolk androgens between species and the variation in within-clutch patterns of yolk androgens between species and investigate whether certain environments or life history traits favor maintenance of higher plasticity or genetically-based variation than others and whether this variation also correlates with variation in other maternal effects involved in creating brood competitive hierarchies such as hatching asynchrony and predictability of the environment. Nevertheless, until now most studies in the field of hormone-mediated maternal effects in birds have focused on functional or evolutionary aspects and the next major advances in our understanding are likely to come from integrating our extensive knowledge of ultimate-level processes with new information about proximate mechanisms.

Figure 1. Linear slope of yolk T concentrations over the egg-laying sequence, from different mothers, from different species. Slope values shown separately for mothers given a control (high quality) diet and a low quality diet for *Serinus canaria*, and mothers with a bold and shy personality for *Parus major*. Unpublished raw data provided by: 1) W. Müller; 2) N. von Engelhardt; 3) C. Carere; 4) R. Safran; 5) A. Raven; 6) T.G.G. Groothuis; 7) D. Gil; 8) G. Johnston; 9) V.C. Goerlich.

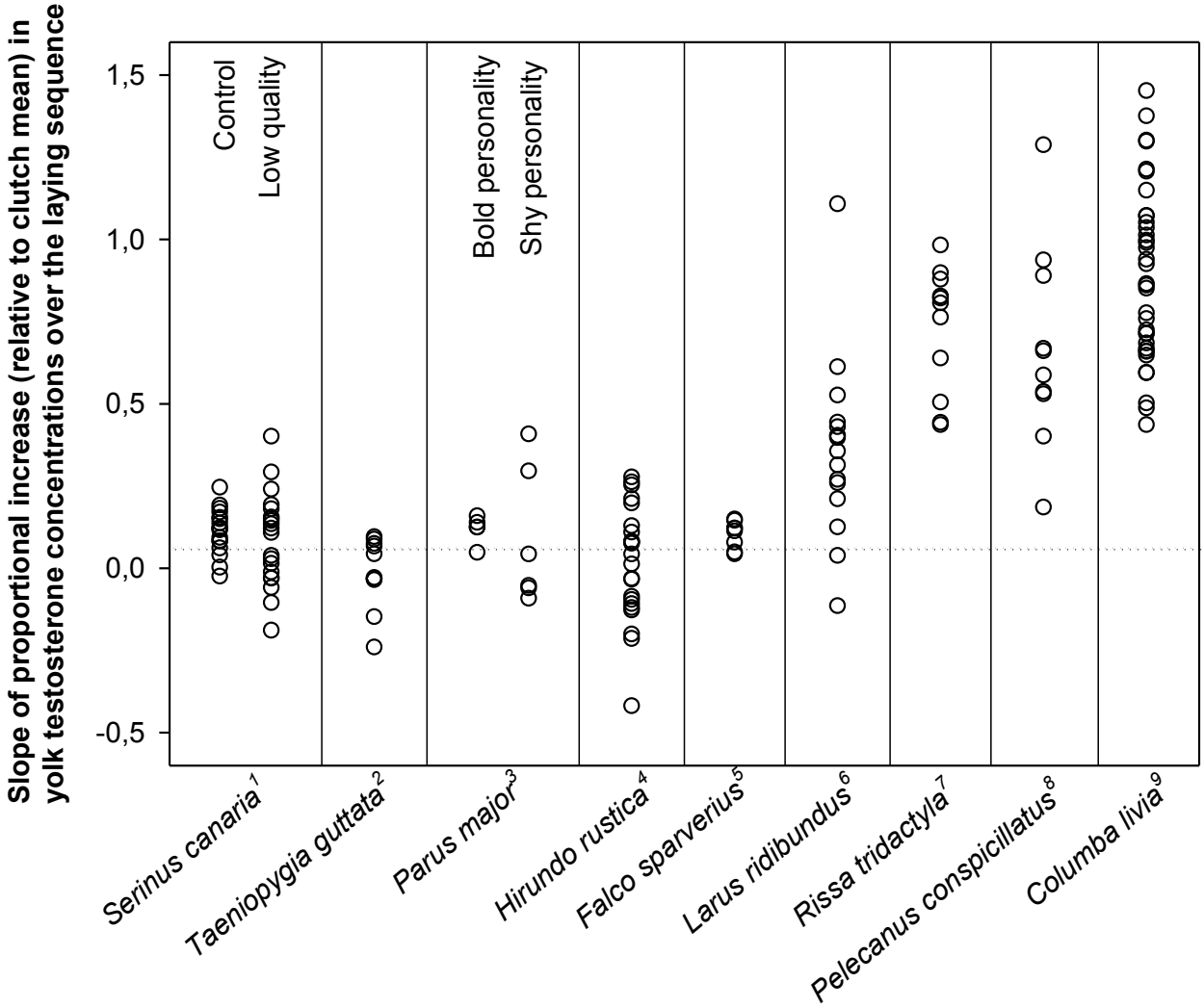
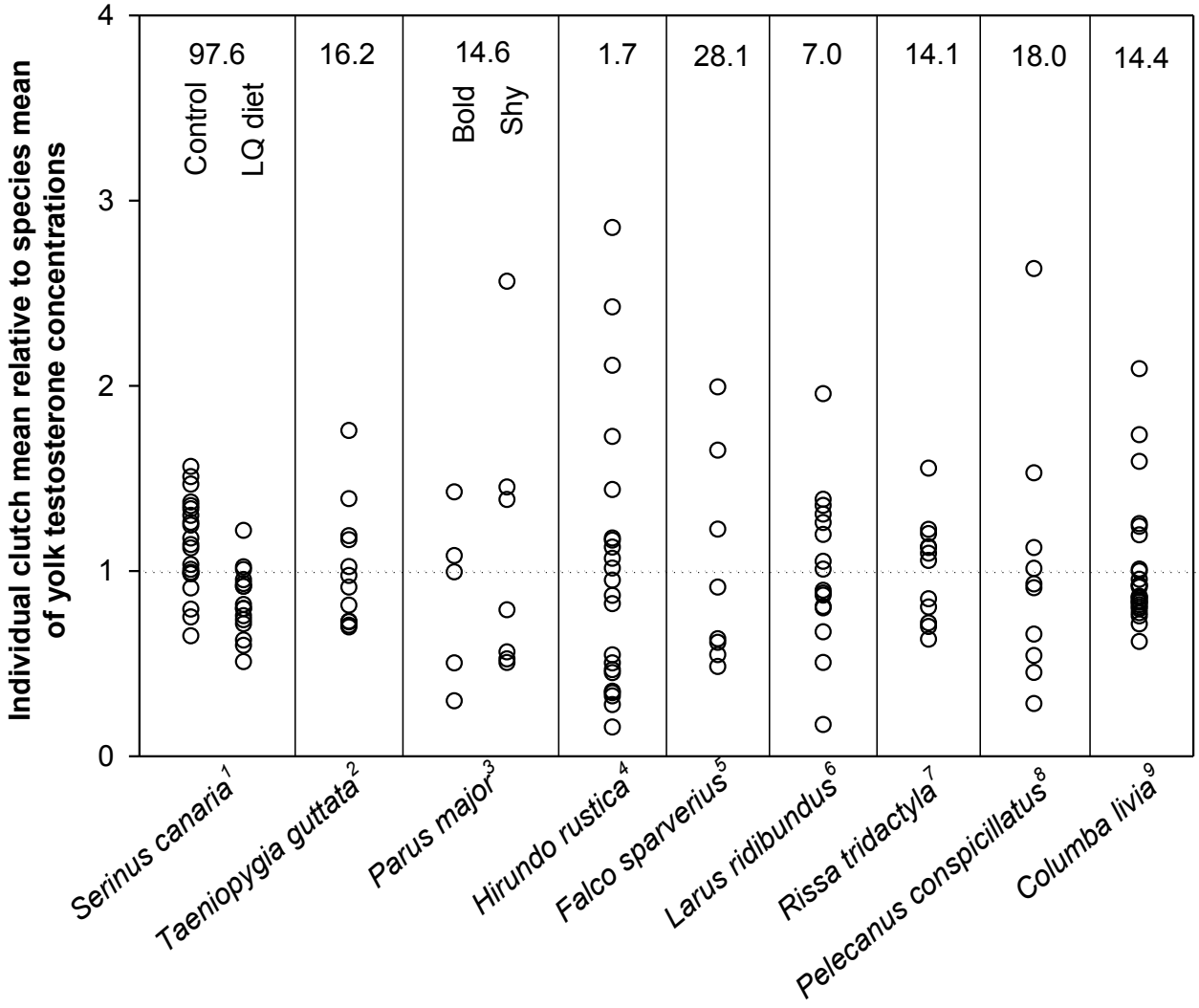


Figure 2. Clutch average yolk T concentrations relative to species mean, from different mothers, from different species. Species mean yolk T concentrations shown at the top of figure. Values shown separately for mothers given a control (high quality) diet and a low quality diet for *Serinus canaria*, and mothers with a bold and shy personality for *Parus major*. Unpublished raw data provided by: 1) W. Müller; 2) N. von Engelhardt; 3) C. Carere; 4) R. Safran; 5) A. Raven; 6) T.G.G. Groothuis; 7) D. Gil; 8) G. Johnston; 9) V.C. Goerlich.



Chapter 2. Selection on the mother or the egg: A comparative analysis of avian maternal testosterone in plasma and yolk

Unpublished manuscript

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Abstract

Hormone-mediated maternal effects have generated much attention as a possible pathway for adaptive non-genetic inheritance. Very little is known about how hormone levels in the circulation of the mother relate to embryonic exposure to these hormones and if such a relationship exists, whether it is due to proximate-level or ultimate-level processes. In many oviparous vertebrate species, breeding females produce testosterone (T) in their ovaries and transfer substantial quantities into egg yolks. At the same time, a considerable fraction of ovarian T also enters the mother's circulation and influences her behavior and physiology. The effects of both yolk T for the offspring and female plasma T for female functioning has been extensively studied in bird species, which we therefore took as our model species. In a phylogenetically-controlled inter-species correlation, we show a significant positive relationship between yolk T and maternal plasma T, which depended on the inclusion of clutch size in the model (reflecting the number of T-producing follicles). Clutch size also correlated positively with maternal plasma T. These results suggest that yolk T and female T are linked at the proximate level, indicating a potential for conflict in selection for optimal maternal T transfer to eggs vs. the mother's plasma. We therefore investigated the degree to which selection might have favored T production and allocation to accommodate offspring needs vs. maternal needs by performing additional comparative analyses in which we correlated several life history traits with both yolk T and maternal plasma T. Coloniality correlated directly with yolk T but not with maternal plasma T, in line with previous work showing that a high yolk T level benefits offspring via early organizing effects on competitive capacity. We also found that species with shorter incubation periods have lower yolk T concentrations. As species evolve short embyronic developmental periods when exposed to high predation rates, they are also likely to benefit from lower yolk T levels as high yolk T exposure enhances conspicuous begging displays in young, which attracts predators to the nest. We also found that in these species, males, whose T production is not constrained by T deposition into yolks, have relatively higher plasma T, which is known to increase duration of both on and off bouts of incubation, and might adaptively limit the frequency of nest visits to avoid leading predators to the nest. We find a similar relationship for females but much weaker. As T has the same effects on incubation behavior in females, this suggests that strong selection on maternal T production for yolks causes female plasma T to reflect a compromise between opposing selection pressures for the offspring and for the mother.

Introduction

Until recently, the development of evolutionary theory was based purely on the Mendelian genetic framework, but this gene-centered view is now expanding to encompass also non-genetic forms of inheritance as empirical and theoretical evidence accumulates for their strong influences on the course of evolution (Day and Bonduriansky 2011). Non-genetically transmitted maternal factors such as hormones can serve as vehicles for the adaptive transmission of plastic, acquired maternal phenotypic traits and information about the current environment (Marshall and Uller 2007). Maternal hormones transferred to seeds (Donohue 2009), eggs (insects: Mousseau and Dingle 1991, birds: Schwabl 1993, reptiles: Bowden et al. 2000, fish: Schaafsma and Groothuis 2012), or directly into the embryos' circulation (Cottrel and Seckl 2009), influence, via phenotypic plasticity, how the information encoded in inherited genes affects the offspring's development, altering their phenotypes and their ability to survive and reproduce in the environment they are born into (Day and Bonduriansky 2011).

Hormone synthesis is a very flexible aspect of the mother's phenotype, changing substantially and rapidly in response to alterations in environmental circumstances or the mother's condition (Nelson, 2005). Variation in the amount of maternal hormones passed on to the offspring can therefore potentially provide information about the mother's phenotype or surroundings at the time of hormone transfer. For example, avian egg yolks were discovered to contain high concentrations of maternal androgens (Schwabl 1993), that vary widely within clutches, between clutches, between mothers and also between species (reviews in Gil 2008, Groothuis et al. 2005, von Engelhardt and Groothuis 2011) generating much interest in the proximate and ultimate causes behind this variation and how it is interpreted by developing young. Since then, many of the advances in the field of hormone-mediated maternal effects have come from studies of birds, as maternal hormones are easily measured and manipulated in the relatively large sealed eggs that develop outside of the mother's body. Maternal androgens enter the yolk during the week(s) of rapid yolk accumulation that precede ovulation (Gill 2007), from the surrounding steroidogenic follicle wall in the mother's ovary (Johnson 2002). After the egg has been laid, the avian embryo gradually assimilates yolk throughout its development via a network of blood vessels that ensure continuous input of maternally-derived substances including androgens, until hatching (Romanoff 1967, Vleck, 1991). Pre-natal exposure to maternal androgens has measurable effects on young that persist well beyond the time at which maternal hormones have been cleared from the chick's circulation (reviewed in von Engelhardt and Groothuis 2011) and even into adulthood (e.g. Eising et al 2006, Rubolini et al. 2007, Strasser and Schwabl 2004, Tschirren et al. 2007, Tobler and Sandell 2007). The most well-studied maternal androgen, testosterone (T), has been shown to increase competitiveness such as territorial (e.g. Müller et al. 2009) or siblicidal aggression (Müller et al. 2012), begging (e.g. Eising et al. 2003), pre- and post-natal growth (Eising et al. 2001), metabolism (Tobler et al. 2007) and to affect several other physiological parameters in young of many species including immune defense as well as androgen production and receptors for androgens (von Engelhardt and Groothuis 2011, Pfannkuche et al. 2011). In addition, in the last decades, substantial experimental work has addressed whether inherited maternal hormones modify offspring phenotype in an adaptive way (reviewed in von Engelhardt and Groothuis 2011) and several comparative studies analyzed inter-species variation in maternal hormone transfer searching for correlations between species shifts in yolk T concentrations and changes in their life histories (Gorman and Williams 2005, Garamszegi et al. 2007, Gil et al 2007, Schwabl et al 2007, Müller and Groothuis 2013).

Not all of the T produced by breeding females in the ovary is absorbed by the yolk - a considerable fraction also enters the mother's bloodstream (Egbert et al. 2013, reviewed in Groothuis and Schwabl 2008) and can therefore influence the mother's physiology and behavior, as female birds have been found to be sensitive to the actions of T throughout the breeding season (reviewed in Ketterson et al. 2005, Rosivall 2013). Circulating T in females increases competitive behavior (Adkins-Regan and Ascenzi 1987, Searcy 1988; Adkins-Regan 1999, Sandell 2007) required for acquiring a mate and/or breeding territory (Veiga and Polo 2008),

and increases singing (Nespor et al. 1996; Adkins-Regan 1999), courtship behaviour (Lank et al. 1999) and social status (Veiga et al. 2004, Sandell 2007). But elevated T in females can also reduce sexual attractiveness (Ketterson et al. 2005), mate choosiness (McGlothlin et al. 2004), fecundity (Rutkowska et al. 2005, Lopez-Rull and Gil 2009, Veiga and Polo 2008), delay egg-laying (Searcy 1988, Clotfelter et al. 2004, Rutkowska et al. 2005), reduce incubation (Rosivall 2013), hatching success (Rosivall 2013), chick-feeding rates (Veiga and Polo 2008), and the number of chicks they succeed in rearing (Lopez-Rull and Gil 2009) and compromise immune function (Duffy et al. 2000, Eens et al. 2000, Peters et al. 2002, Casto et al. 2001, Mougeot et al. 2004), indicating that elevated T might mediate trade-offs in energy allocation towards different aspects of breeding behavior and physiology. Comparative studies show that peak female T is higher in monogamous species than in polygamous species (Ketterson et al. 2005, Møller et al. 2005), higher in colonially-breeding species than in solitary breeders (Møller et al. 2005, but see Ketterson et al. 2005), and relatively lower compared to peak male T in sexually dichromatic species (Ketterson et al. 2005) suggesting that significant variation in female plasma T concentrations stems from co-evolution with other life history traits. These studies also showed that female peak T directly correlates with male peak T concentrations (Ketterson et al. 2005, Møller et al. 2005) indicating that male and female T are partly the direct product of the same selection pressures, or that variation in female T reflects a correlated response to strong selection on male T concentrations.

Despite relatively thorough separate investigations of the functions and potential adaptive nature of maternal T for offspring and mothers, no comparative study has yet tested whether they co-vary. Such an analysis would shed light on whether maternal T transfer to eggs is a phenotypic trait on which selection can act independently or whether it is physiologically linked with maternal plasma T concentrations which mediate important functions in the mother herself and may therefore limit T deposition in the egg (Groothuis and Schwabl 2008). If maternal plasma T and yolk T do not co-vary, for example, this would indicate that maternal plasma T and yolk T can be independently regulated and that these concentrations have diverged over evolutionary time in response to different selective regimes (Groothuis and Schwabl 2008). A positive relationship between maternal plasma T and yolk T would indicate that either no mechanism has yet evolved to permit their independent regulation (physiological epiphenomenon hypothesis, Groothuis and Schwabl 2008), or maternal plasma T and yolk T are under identical selective regimes. A negative relationship might indicate that high amounts of ovarian T can be shunted into the yolk or into the mother's plasma but not into both simultaneously, creating a negative correlation (flexible distribution hypothesis, Groothuis and Schwabl 2008) or selection for high T in the offspring would be accompanied by selection for low T in the mother. Here we perform the first comparative study that correlates species averages of maternal plasma T and yolk T during the egg-laying period.

To distinguish whether correlations between yolk T and female plasma T have arisen simply due to the mechanism of hormone distribution from the ovary or due to correlated selective regimes, we investigated the life history traits that have co-evolved with maternal T in the mother's plasma and in the egg, compare and contrast the suites of predictive life history traits for plasma T and yolk T, and discuss how selection might have shaped the interspecies patterns in maternal T in the mother's plasma vs. in the egg. We identified the traits that were the most important predictors in several previous comparative analyses of female breeding plasma T (mating system [Ketterson et al. 2005, Møller et al. 2005], sexual dichromatism, and coloniality [Møller et al. 2005]) and yolk T (incubation and nestling periods in passerine birds, Gorman and Williams 2005; Schwabl et al. 2007) and for the first time use them to predict both maternal plasma T during the egg-laying period and in a separate analysis to predict maternal T in yolks. We also analyze male plasma T levels during the egg-laying period as they represent members of the same species that are not exposed to potential constraints imposed by T deposition in yolks. While previous comparative studies of female plasma T performed their correlations on peak female T levels (Ketterson et al. 2005, Møller et al. 2005), which occurred mostly during the pre-laying period (Ketterson et al. 2005), this is the first study to analyze female plasma T and male plasma T during the egg-laying stage.

Methods

Data collection

We extracted data of plasma T concentrations during the egg-laying period for males and females from a comparative study performed by Ketterson et al. (2005) and supplemented this set of values with additional data from recent primary literature and from unpublished data from colleagues and our own lab. We were able to collect plasma T data for 38 species coming from 28 families and 14 orders. We also extracted species averages of yolk T concentrations from several comparative studies (Gorman et al., 2007; Gil et al. 2007, Schwabl et al. 2007, Garamszegi et al. 2007), and updated them with recently published papers and with data from colleagues and our own lab. We collected yolk T data for 138 avian species from 51 families and 20 orders.

For the species in our dataset, we also collected information about mating system, sexual dichromatism, coloniality, incubation period, nestling period and clutch size from the above cited comparative studies, from databases and handbooks (Cramp 1998; Poole 2005; de Magalhaes and Costa 2009), the primary literature, or our own laboratory data (raw data are available upon request).

If estimates for certain T concentrations or continuous life history traits (e.g. nestling period or incubation period) were available from several sources, we averaged all values. Mating system was coded as a binary score of 0 for monogamous species and 1 for polygamous species. Sexual dichromatism was coded as 0 for monochromatic species and 1 for sexually dichromatic species (species in which plumage and other ornaments were sexually dimorphic). Coloniality was coded as 0 for solitary species breeding in large all-purpose territories and 1 for species breeding in aggregations in which breeding pairs have just small territories containing a nest site.

Phylogenetic analyses

We performed all of our phylogenetic least squares regressions (PGLS) in the R statistical computing environment (R Development Core Team 2012) using the "ape" package (Paradis et al. 2004) to translate the topology of our phylogenies into a variance covariance matrix, and "nlme" to perform the regressions that incorporated the phylogenetic information (Pinheiro et al. 2010).

We constructed composite phylogenies for our three datasets (app. 1-3) from several recent molecular bird phylogenies (Friesen and Anderson 1997, Pons et al. 2005, Bridge et al. 2005, Sheldon et al. 2000, Alstrom et al. 2006, Lanyon and Omland 1999, Kan et al. 2010, Jønsson and Fjeldsa 2006, Hackett et al. 2008). Because our phylogeny contained parts from several different published trees, we set all distances between nodes to equal length. This approach has been demonstrated to be the most conservative (Garland et al. 1993; Pagel 1994; Purvis et al. 1994), and different branch lengths do not alter results qualitatively (Poiani and Pagel 1997; Møller et al. 1998; Nunn 1999; Poulin 1999).

PGLS regressions correct for phylogenetic nonindependence of data coming from related species by incorporating information about their shared evolutionary history via a variance covariance matrix. The regression estimates λ, the off-diagonal multiplier of elements of this matrix, via likelihood ratio statistics to best fit the distribution of the data to the tips of the phylogeny (Diniz-Filho et al. 2012, Hernandez et al. 2013). λ, the values of which can range between 0 and 1, represents the degree to which traits have evolved according to a Brownian process. In a Brownian model of trait evolution, traits evolve along the branches of a phylogeny via a random walk, and trait differences accumulate over time, so that as lineages diverge, traits are more similar within clades than they are between clades merely due to stochastic evolutionary processes (Freckleton et al. 2002, Cooper et al. 2010). A λ value close to 0 indicates that a trait has evolved independently of phylogeny whereas a λ value close to 1 indicates that

patterns of trait evolution are consistent with evolution according to Brownian motion. The statistical package we used in R did not produce residuals corrected for phylogeny, so we present phylogenetically uncorrected (raw) data in figures.

Statistical models

Female plasma T, male plasma T, yolk T, incubation period and nestling period all exhibited a log-normal distribution and were therefore log-transformed in all analyses. We tested whether maternal plasma T correlates with yolk T using a dataset including 17 species (see app. 1 for phylogeny) for which we had data for yolk T and plasma T concentrations in breeding females. Because each yolk is produced within an ovarian follicle (the main site of T production in females) and because yolks contain much higher T concentrations than the plasma does and are directly adjacent to the site of T synthesis, we used yolk T levels as the predictor and female plasma T levels as the response variable in this model. Also, clutch size varies substantially among these species and influences the number of hierarchical follicles producing maternal T affecting female plasma levels, so we included clutch size as a second predictor in this model. In the first PGLS model we included yolk T and clutch size as predictors of female plasma T concentrations. We then repeated this model but excluded clutch size to analyze the importance of this predictor for the model. Reversing the position of yolk T and plasma T in the models did not change the outcome qualitatively (results are not shown). We also tested whether female plasma T during the egg-laying period correlates with male plasma T during the egg-laying period.

In the analyses that follow, we tested whether aspects of the species' life histories predicted T concentrations in the plasma or the yolk. We performed five sets of analyses, each testing the correlation of female plasma T, male plasma T and yolk T with one of five life history species traits found to be important predictors in previous comparative studies: mating system, sexual dichromatism, coloniality, incubation period, and nestling period.

Each set of analyses began with a phylogenetic correlation between the life history trait of interest and female plasma T. As the aim was to determine whether these changes in life histories directly influenced maternal plasma T concentrations, we did not include clutch size as a predictor as we had in the previous set of analyses. We then performed a phylogenetic correlation between the same trait and male plasma T. If both correlations were significant this suggests that plasma T averages shifted in response to a change in life history in a similar way for both sexes of the same species. If only one of the correlations was significant, this would indicate some degree of sexual divergence in plasma T in response to evolution of a life history trait, which may be due to additional selection pressures on females that don't exist for males, for example selection on yolk T allocation. Third and finally, we performed a phylogenetic correlation testing the association between yolk T and each of the five species traits of interest. Similar to our comparisons in the life history correlations with female T and male T, we compare the correlations between female T and yolk T to assess whether they have shifted in response to changes in life histories in similar ways or whether they have diverged. All of our analyses were two-tailed with a 95% confidence level.

To facilitate comparison of outcomes from different models we calculated effect sizes for each of the life history trait correlations using the formula for "Cohen's d" and 95% confidence intervals provided in Nakagawa and Cuthill (2007). Effect sizes from models containing a continuous predictor were first calculated as "r" and then converted to "Cohen's d" via a formula provided in Nakagawa and Cuthill (2007).

Results

Maternal T in yolk and plasma

Yolk T directly correlated strongly and positively with maternal plasma T during the laying period (table 1, fig. 1A). In the same model, clutch size also showed a positive correlation with

maternal plasma T at laying (table 1, fig. 1B). In a model in which clutch size was not included, the predicted value of yolk T for variation in female plasma T lost its significance (p = 0.066), suggesting that the number of hormone producing follicles is part of the mechanism determining female circulating T concentrations.

	b	SE	t	p	λ	n
Yolk T	0.859	0.197	4.352	**0.001**	<0.01	17
Clutch size	0.243	0.102	2.384	**0.032**		
Yolk T	0.490	0.247	1.983	0.066	<0.01	17
Male T	0.444	0.117	3.788	**0.001**	0.23	38

Table 1. Results from phylogenetic least squares regressions predicting female plasma T concentrations during egg-laying. Results are shown for three models: 1) including average yolk T concentrations and average clutch size, 2) including only average yolk T concentrations, 3) including male T concentrations during the egg-laying phase. λ indicates degree of phylogenetic dependence in each regression. T variables are log-transformed.

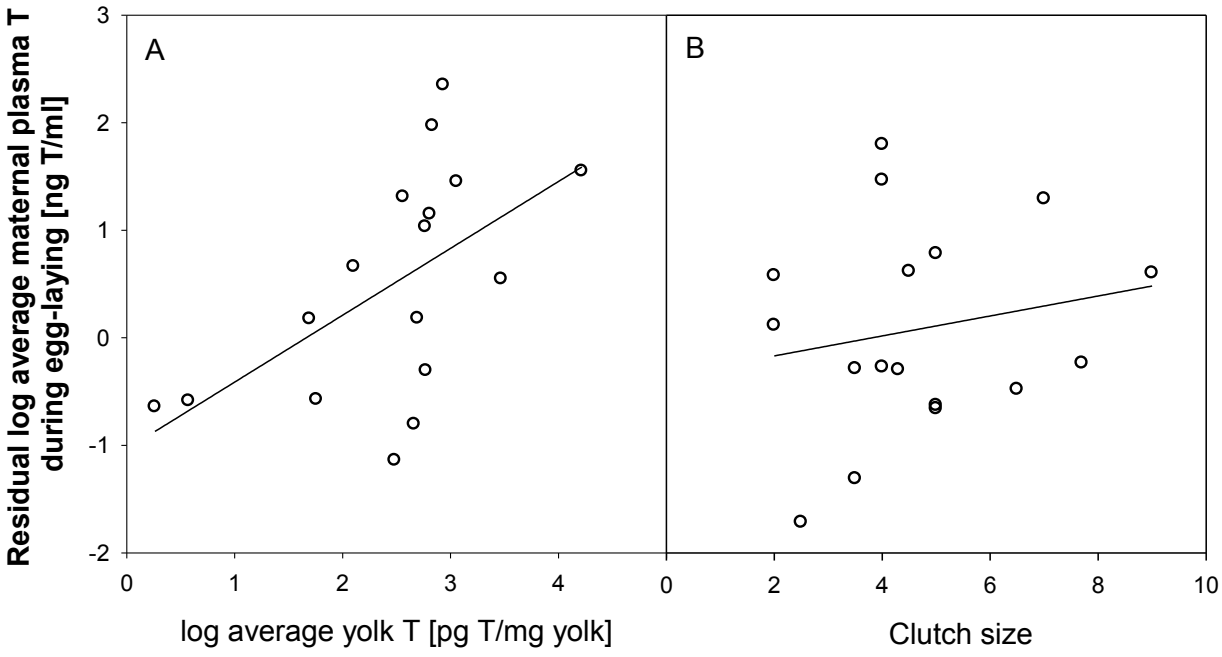


Figure 1. Phylogenetically uncorrected residual species averages of maternal plasma T during egg-laying stage from a model including species averages of maternal T in yolks (A) and clutch size (B). All T variables are log-transformed.

Maternal plasma T and paternal plasma T

Female plasma T during the laying period correlated strongly and positively with male plasma T during the laying period (table 1, fig. 2).

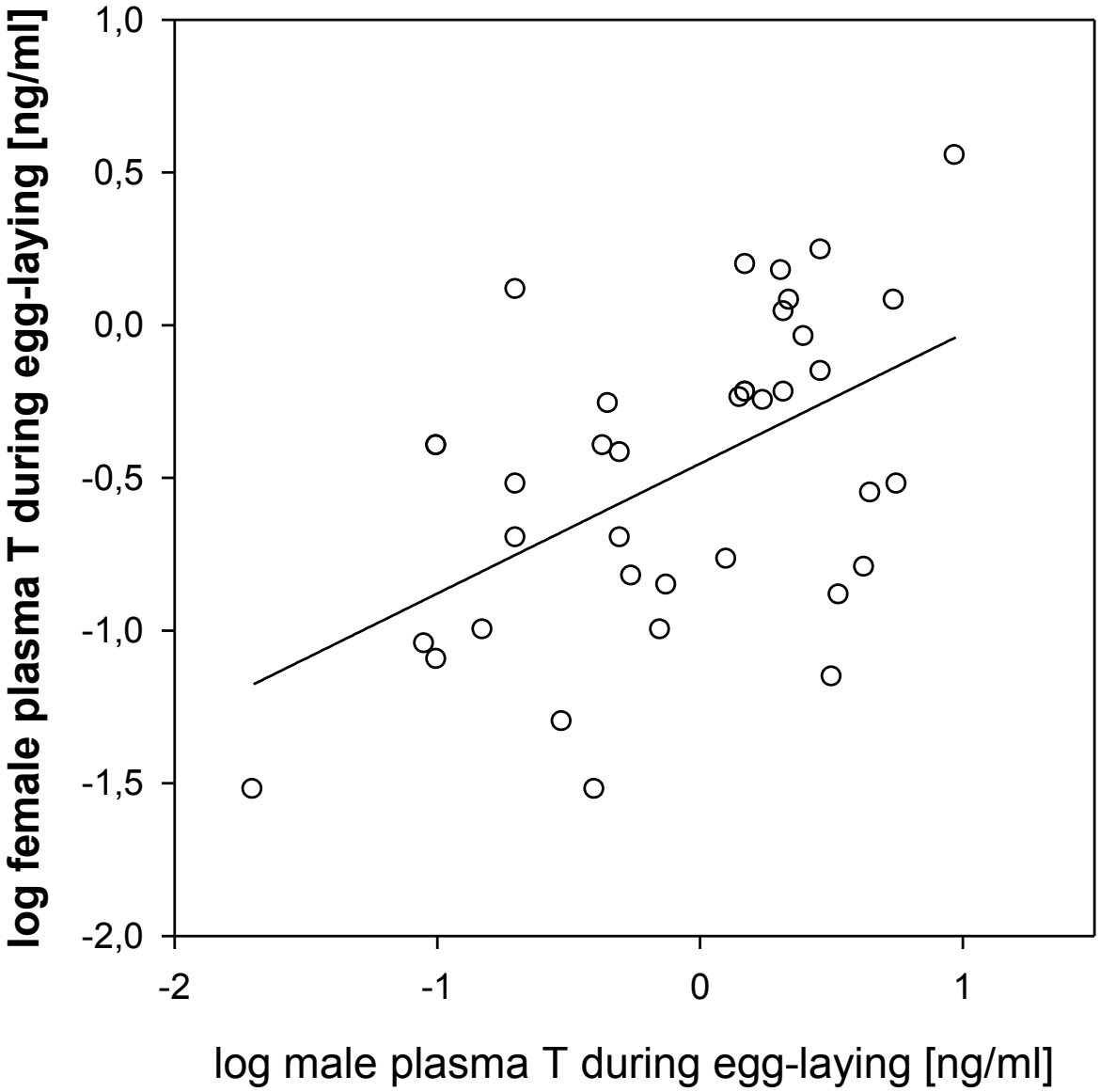


Figure 2. Phylogenetically uncorrected species averages of maternal plasma T during egg-laying stage in relation to paternal plasma T during egg-laying stage (n = 38). Both variablesare log-transformed.

Correlations between T and life history traits

Mating system, sexual dichromatism and nestling period were not correlated to plasma T of females, plasma T of males or with yolk T (table 2, figs. 3A, 3B, 3E). Coloniality was not significantly related to female or male plasma levels (table 2, fig. 3C), but colonial species have higher yolk T concentrations than do solitary species (table 2, fig. 3C).

Incubation period was positively correlated with yolk T concentrations (table 2, fig. 3D), negatively correlated with male plasma T concentrations and showed no significant relation with female plasma T concentrations (table 2, fig. 3D).

	b	SE	t	p	λ
Predictor: Mating system					
Female plasma T	-0.111	0.436	-0.254	0.801	<0.01
Male plasma T	0.437	0.520	0.840	0.407	0.30
Yolk T	-0.285	0.181	-1.573	0.118	0.79
Predictor: Sexual dichromatism					
Female plasma T	0.031	0.414	0.076	0.940	0.22
Male plasma T	0.176	0.496	0.355	0.725	0.32
Yolk T	0.270	0.156	1.730	0.086	0.82
Predictor: Coloniality					
Female plasma T	0.113	0.381	0.297	0.768	<0.01
Male plasma T	-0.515	0.481	-1.070	0.292	0.29
Yolk T	0.381	0.160	2.384	**0.019**	0.80
Predictor: Incubation period					
Female plasma T	-0.703	0.515	-1.365	0.181	0.50
Male plasma T	-1.652	0.578	-2.857	**0.007**	0.52
Yolk T	0.667	0.301	2.217	**0.028**	0.75
Predictor: Nestling period					
Female plasma T	0.152	0.161	0.948	0.350	<0.01
Male plasma T	-0.225	0.285	-0.791	0.434	0.49
Yolk T	0.112	0.105	1.066	0.288	0.79

Table 2. Results from phylogenetic least squares regressions of underlined life history traits predicting outcome variables: female plasma T, male plasma T and yolk T. Analyses performed on female and male plasma T include 38 species, analyses performed on yolk T include 138 species. λ indicates degree of phylogenetic dependence in each regression. T variables, incubation period and nestling period are log-transformed.

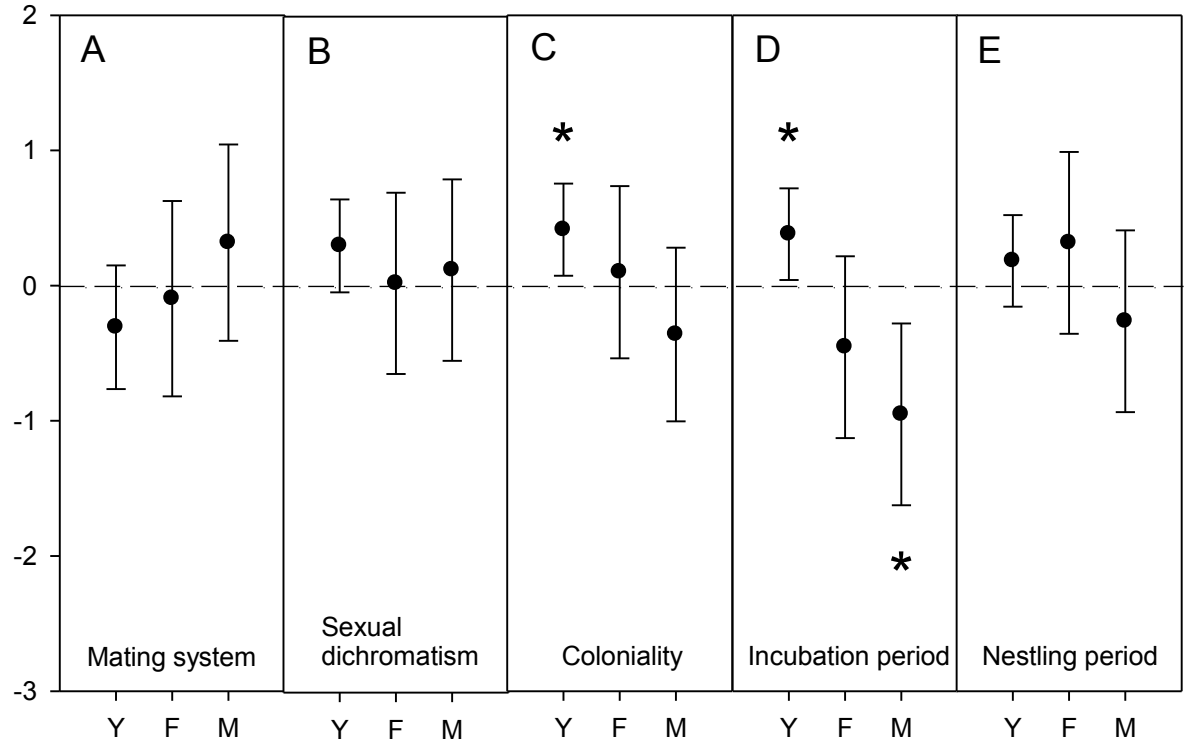


Figure 3. Effect sizes (Cohen's d) and 95% CI for phylogenetic correlations from models in which life history traits predict the following T parameters: "Y" refers to yolk T concentrations, "F" refers to female plasma T concentrations during the egg-laying stage, "M" refers to male plasma T concentrations during the egg-laying stage. Analyses were performed on n = 38 species for male and female plasma T, n = 138 species for yolk T. For panel A, species were classified as 1 = monogamous, 2 = polygamous. For B, species were scored as sexually dichromatic or not (1 or 0). In C, species were scored as breeding colonially (1) or solitarily (0). In D, incubation period represents the average days between oviposition and hatching. In E, nestling period represents the average days between hatching and leaving the nest. Effect sizes in which confidence intervals do not cross "0" are statistically significant. T variables, incubation period and nestling period were log-transformed. Positive values indicate positive correlations and negative values indicate negative correlations.

Discussion

One of the main aims of this study was to investigate the degree to which variation in the hormone concentrations that the mother transfers to the offspring is also reflected in the mother's circulation. As birds are frequently studied in the field of hormone-mediated maternal effects, we collected data from a diverse set of avian species and performed the first inter-species correlations of average maternal plasma testosterone (T) during the egg-laying period and average yolk T. We discovered that mean species values of yolk T concentrations correlated positively with mean species values of maternal plasma T concentrations, especially when the number of hormone producing follicles, as estimated by clutch size, are taking into account. On a proximate level, this is to be expected as T is produced in individual follicles so that a larger number of active follicles, and thus a larger clutch size, would lead to higher concentrations of circulating T in the mother but not in each individual egg. The fact that incorporating clutch size is so important for revealing a direct relationship between yolk T and maternal plasma T suggests that ovarian T synthesis is reflected both in the yolk and the mother's blood and a mechanism has not yet emerged to regulate T levels in female plasma and yolk independently, either because of physiological constraints or lack of strong enough selection pressure. If the

former is the case, this creates the potential for antagonistic selection between optimal hormone exposure for the offspring vs. the mother.

Previous intra-species correlations between yolk T and maternal plasma T during egg production have failed to consistently produce a positive correlation: some studies show a positive correlation (canary, Schwabl 1996; house finch, Badyaev et al. 2005; tree swallows, Whittingham and Schwabl 2002, black-headed gulls, T. Groothuis, unpublished data; chicken, R. Henriksen, unpublished data) but many show a negative correlation (black-backed gull, Verboven et al. 2003; house finch, Navara et al. 2006; house sparrow, Mazuc et al. 2003), or no correlation (pigeon, Goerlich et al. 2009; canary, Tanvez et al. 2004; group level: starling, Pilz et al. 2004; canary: Marshall et al. 2005; reviewed in Groothuis and Schwabl 2008). There are two major difficulties with these within-species correlations. First, as mentioned above, maternal plasma T reflects the cumulative T production of all follicles (therefore is expected to vary with clutch size), whereas yolk T reflects mostly T production in the single follicle that surrounds it. Second, in addition to potential socially-induced short-term T fluctuations, maternal plasma T fluctuates considerably due to the egg-formation process with pre-ovulatory T levels rising to 2-3 times baseline values for ca. 8-10 hours (Yang et al. 1997, Yang et al. 2005, Johnson and Tienhoven 1980, Doi et al. 1980) and thereafter returning to baseline concentrations, so a single sample of plasma T is likely to provide a very imprecise measure of the mother's average plasma T concentrations during that breeding stage. Yolk T concentrations, on the other hand accumulate over several days and reflect the total ovarian T production over the course of the yolk formation period (Bahr et al. 1983, Lipar et al. 1999, Hackl 2003, Okuliarova et al. 2010).

Despite the evidence that proximate-level processes might be responsible for creating this positive correlation, we investigated it also on an ultimate level by analyzing whether it might have emerged due to identical selective regimes on the mother and the egg. The positive correlation between maternal plasma T and yolk T contained substantial unexplained variation, which can arise from differences in transfer efficiency of T into the yolk, enzymatic conversion of yolk T to other metabolites, transfer efficiency of T to the blood, or hormone clearing rates from the mother's plasma. These additional sources of variation permit statistical analyses exploring whether ovarian T production has evolved to benefit the mother or the egg, or both. For several life history traits, selected from the literature on maternal hormones in birds, we therefore compared the strength of the correlation between maternal plasma T *versus* yolk T. In addition, given the strong positive correlation that we found between female plasma T and male plasma T (fig. 2), we compared the strength of correlations between life history traits and maternal plasma T with the strength of the correlations with paternal plasma T to assess whether selection pressures on females resemble those on males more than they resembles those on eggs.

In contrast to previous comparative studies on peak female plasma T concentrations, none of the five life history traits that we analyzed correlated with female plasma T concentrations during the egg-laying period, but two of the traits (incubation period and coloniality) showed significant positive correlations with yolk T concentrations. Male T correlated negatively, rather than positively with one of these (incubation period). Below, we discuss the underlying selection pressures that are likely to have created these significant relationships.

Coloniality

Møller et al. (2005) found that peak female plasma T levels were higher in species breeding at higher densities ("colonial") birds than solitary birds. Within-species correlations have shown that males from larger colonies have larger testes (Brown and Brown 2003), and circulating T in both males and females correlate positively with colony size (Smith et al. 2005) as well as breeding density both in colonial and solitary breeders (Ball and Wingfield 1987, Beletzky et al. 1990, 1992; Wingfield and Hahn 1994). This is in line with the findings of several studies showing that T mediates aggression in adult colonial (Alonso-Alvarez and Velando 2001) and non-colonial breeders (reviewed in Soma 2006) and facilitates their acquisition of larger

territories (Alonso-Alvarez and Velando 2001, Wingfield 1984, Moss et al. 1994). But peak T levels more often than not occur during the pre-laying period when territories are being established, males and females engage in courtship and construct nests (e.g. Ketterson et al. 2005, Wingfield et al. 1987) and these levels decrease substantially at the onset of egg incubation when social relationship have been established. Therefore, not surprisingly, our interspecies analyses found no evidence that average species plasma T levels during the egg-laying stage were higher in colonially-breeding females or males, than in solitary species.

We discovered, however, that colonial birds produce eggs containing higher concentrations of yolk T than do solitary breeders (fig 3). A positive relationship between breeding density and yolk androgens has also been found within many avian species (e.g. Pilz and Smith 2004, Eising et al. 2008, Schwabl 1997, Whittingham and Schwabl 2002, Safran et al. 2010, Remes 2011, van Dijk et al. 2013). Exposure to elevated yolk T enhances territorial and competitive behavior in colonial chicks that can be important for rapidly accessing and defending limited food delivered by parents and defensive action against nest territory intruders (Müller et al. 2009, Müller et al. 2012) and potentially for soliciting food from unrelated parents or becoming adopted in foreign broods (Müller et al. 2012). The stronger relationship between coloniality and yolk T compared with maternal plasma T suggests that the evolution of aggregated breeding selected ovaries to produce more T to accommodate offspring rather than mothers.

Embryonic developmental period

We found a positive relationship between yolk T concentrations and incubation period. Two previous comparative studies performed on passerine birds, found a negative rather than positive relationship between incubation period and yolk T concentrations (Gorman and Williams 2005; Schwabl et al. 2007) and suggested that high yolk androgen exposure might be the proximate cause behind the faster development rates, although in Gil et al. (2007) this relationship within passerines was no longer statistically significant after correcting for phylogenetic relationships between species, and in a larger analysis including non-passerine species the study found no relationship between yolk T and incubation period. Indeed, past experimental studies show that more often than not, yolk T exposure does not shorten incubation periods (shortened: black-headed gulls, Eising et al. 2001; starlings, Müller and Eens 2009; not shortened: starling, Pilz et al. 2004, yellow-legged gull, Rubolini et al. 2006, penguin, Poisbleau et al. 2012; kittiwakes, Müller et al. 2012; feral pigeons, Hsu et al. chapter 6 of this thesis; lengthened: American kestrel, Sockman et al. 2008; zebra finch, von Engelhardt et al. 2006, Boncoraglio et al. 2011) although most of these studies were performed on non-passerines.

If yolk T concentrations do not consistently influence development rates, why do species with short incubation periods produce eggs with relatively low concentrations of yolk T? We suggest that predation rate might be the actual selection pressure underlying the relationship between yolk T and incubation period because predation rate correlates strongly with incubation period. Extensive previous work on individual species shows that high predation risk leads to faster development rates (reviewed in Martin and Briskie 2009) which fits with findings from comparative studies that species with high predation rates have short incubation periods both in passerines (Schwabl et al. 2007) and non-passerines (Hipfner et al. 2010). High maternal T exposure has been shown to increase begging activity in nestlings, which attracts predators (Leech and Leonard 1997, McDonald et al. 2009) and therefore motivates the expectation that yolk T should be lower in a predator-rich environment. Indeed, a recent study on great tits demonstrated that experimentally increased predation rate significantly decreases yolk T concentrations (Coslovsky et al. 2012). Although Schwabl et al. (2007) found a positive rather than negative relationship between yolk T and predation rate in passerines, our findings performed on a broader dataset that includes both passerines and non-passerines support the idea that species with short incubation periods (indicating a predator-rich environment) are under selection to have lower yolk T concentrations than species with lower predation risk.

One question that remains unanswered is why do males of species with the shortest incubation periods have the highest circulating T? In most avian species, including passerines, incubation begins near the end of the egg-laying period. In a predator-rich environment, incubating parents increase the duration of their bouts of nest presence and nest absence (to minimize the number of times they fly to and from the nest) and this pattern occurs both within (e.g. Ferretti et al. 2005; Kleindorfer 2007; Massaro et al. 2008; Weathers and Sullivan 1989, reviewed in Martin and Briskie 2009) and between species (Conway and Martin 2000). T elevation has been shown to produce exactly this type of incubation pattern (in females, Rosivall 2013) which poses some risk of reduced hatching success if ambient temperatures drop (Rosivall 2013) but the cost of fewer eggs hatching may be offset by the much larger benefits coming from reduced total nest failure rates due to lower predation rates as less visits to the nest makes the nest less conspicuous. The mechanism underlying the widely observed altered incubation behavior that occurs when predation rates are high, has yet to be studied directly, although the effects of elevated plasma T on incubation behaviour certainly make T a very likely candidate (e.g. in males: Alonso-Alvarez 2001, Schwagmeyer et al. 2005, Oring et al. 1989, De Ridder et al. 2000, McDonald et al. 2001, in females: Rosivall, 2013), even though until now it was usually interpreted as a costly side-effect of adaptive plasma T elevation rather than the trait under direct selection. However, an alternative explanation might be that elevated T increases anti-predator defense behaviour. Thus far, this does not seem to be the case, as experimentally elevated T had no effect on nest defense behavior against predators in male juncos (Cawthorn et al. 1988), but it deserves further investigation.

The correlation between incubation period and female plasma T appears to be weakly negative (fig. 3D) and the effect size lies almost exactly between the positive effect size of the correlation with yolk T and the negative effect size of the correlation with male plasma T. We suggested above that high predation risk may be responsible for the two opposing relationships between T and incubation period that we found: in offspring, low yolk T might be beneficial to prevent nest predation, whereas in males, high plasma T might be beneficial in preventing nest predation. If the effect of elevated plasma T on incubation behavior are the same in females, which appears to be the case (Rosivall 2013), then selection on female plasma T to resemble that of males (i.e. high plasma T when predation is high) may be constrained by opposing pressure on maternal T production to produce low amounts of T for developing yolks in that same context, producing a "compromise", i.e. the weak negative relationship that we found in our analyses.

General implications

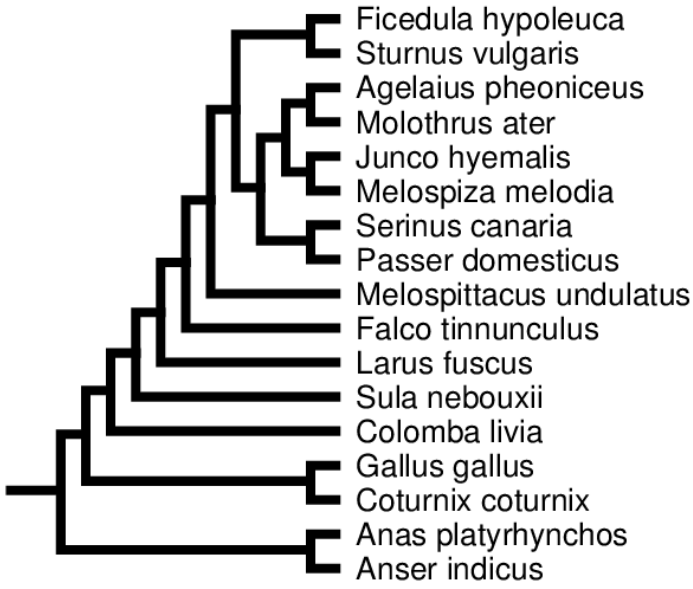
Our findings suggest that the positive correlation between yolk T and female T is more likely due to the fact that they are not independently regulated rather than that they are the product of identical selection pressures. First, clutch size showed a significant positive relationship with female plasma T suggesting that females with a larger number of steroidogenically active developing follicles contain higher concentrations of T in their circulation. Second, we found that the strength of the correlation between yolk T and female plasma T improves when including the number of active follicles that release T into the blood (clutch size), in the model. The importance of clutch size as a moderating variable indicates that female plasma T correlates positively with the concentrations of T in a single yolk and supports the idea that T produced in an active follicle enter the yolk and the mother's circulation without independent regulation. We make this conclusion because if the correlation between female plasma T and yolk T were due to identical selective regimes, selection would have acted directly on female plasma T concentrations and yolk T concentrations creating a positive correlation which should weaken with the inclusion of clutch size in the model, yet we show that inclusion of clutch size strengthens it. Third, female T did not correlate with the same life history traits as yolk T did, suggesting that variation in the two variables are not the product of the same selective regimes. This also suggests that maternal T production in the ovary is driven by factors that appear to primarily accommodate functions in offspring rather than functions in mothers.

We found that coloniality was strongly correlated with yolk T (most likely to increase offspring competitiveness in high density areas) but not with maternal plasma T, (perhaps because high circulating T is no longer beneficial after the most socially competitive period has ended). We also found that in species with short incubation periods (a trait associated with a high predation rate), egg yolks have lower T concentrations, which may reduce conspicuous T-mediated begging displays to avoid attracting predators to the nest. We also found that in these species, males have higher plasma T, which has been shown to alter incubation behavior in a manner that avoids attracting predators to the nest. Incubation period (i.e. predation rate) may represent an example of antagonistic selection on maternal T production in the ovaries, as it might select for high T in the mother's plasma (as seen in males) but low pre-natal T exposure for offspring (as seen in yolks). Yolk T and male plasma T appear to show a similar pattern of opposing relationships to two other life history traits (coloniality and mating system) with the female plasma T correlation falling in the middle, supporting the idea of antagonistic selection, but these effects were not statistically significant.

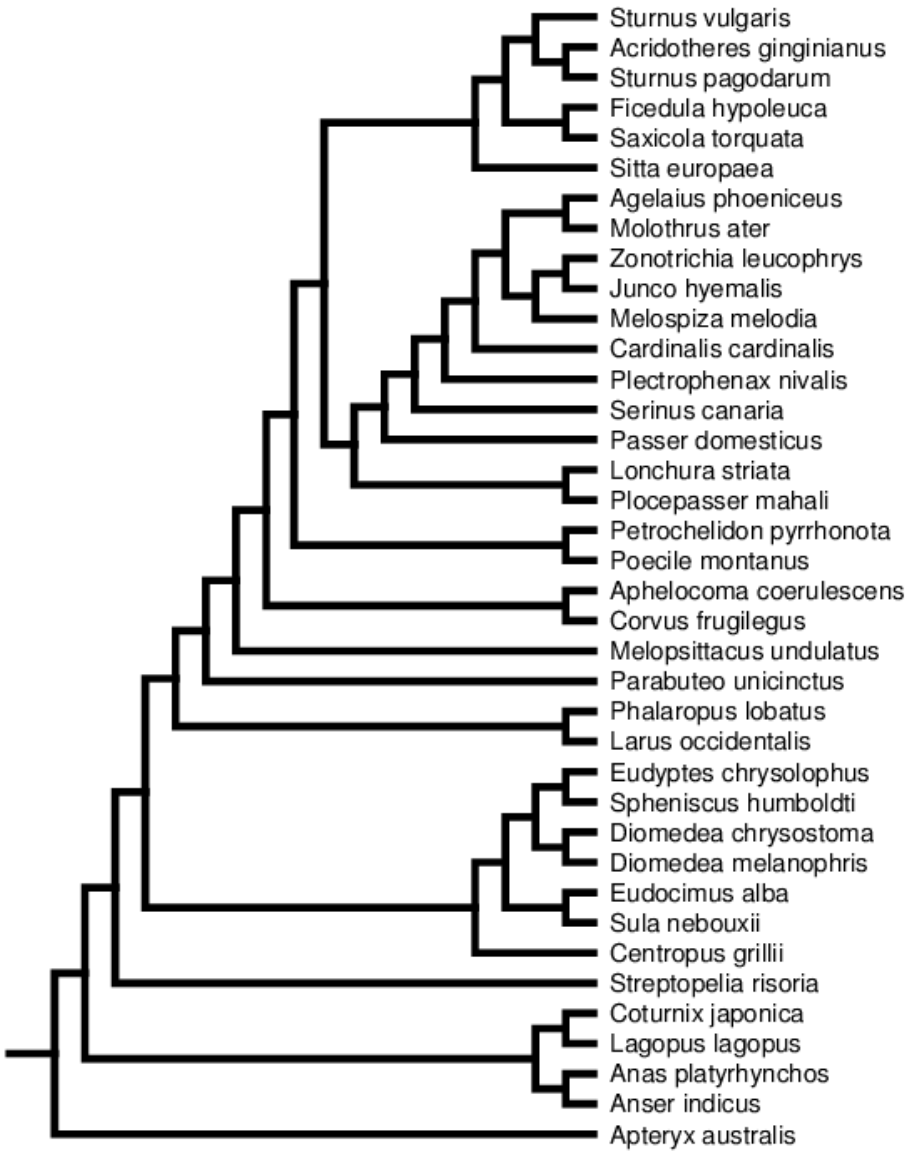
Our ability to distinguish whether selection acted on maternal plasma T or yolk T was limited by the strong correlation between maternal plasma T and yolk T concentrations. Similarly, our ability to distinguish whether selection acted on maternal plasma T or paternal plasma T was limited by their strong correlation. Therefore, if we found a significant association between a life history trait and maternal T in eggs, then some association, even if weaker and not statistically significant, was usually present in the mother (and vice versa) and the same is true when comparing correlations with maternal plasma T and paternal plasma T. For this reason the confidence intervals of the effect sizes of the phylogenetic correlations between individual life history traits and T variables almost always overlapped (Fig. 3). Nevertheless we suggest that comparative analyses of maternal hormones in only females present only part of the story and variation in maternal T production may reflect selection on yolk T and selection on maternal plasma T.

However T concentrations are only one component of the androgenic signaling system, and are only effective to the extent that the mother and offspring are sensitive to their actions. Changes in androgen receptor density, tissue-specific conversion of T to other metabolites, shifts in the amount of binding globulins, and other mechanisms can change the sensitivity of mothers or offspring to the amount of T they are exposed to. If maternal T production is selected to optimize concentrations in the mother's circulation and this imposes costs on offspring, then offspring should evolve mechanisms to adjust their sensitivity to maternal T, for which there is some recent evidence (Pfannkuche et al. 2011) and the opposite should be true if maternal T production is selected to optimize T for the egg (Groothuis et al. 2005). The frequent inconsistencies in the effects of hormone elevation studies (assuming sound methodology) indicate widespread context-dependency of effects and point toward a substantial role for environmental modulation of sensitivity to the hormone, which may release the mother from the potential constraint.

Appendix 1. Topology of the composite phylogeny used in phylogenetic least squares regressions of maternal plasma T and maternal yolk T.

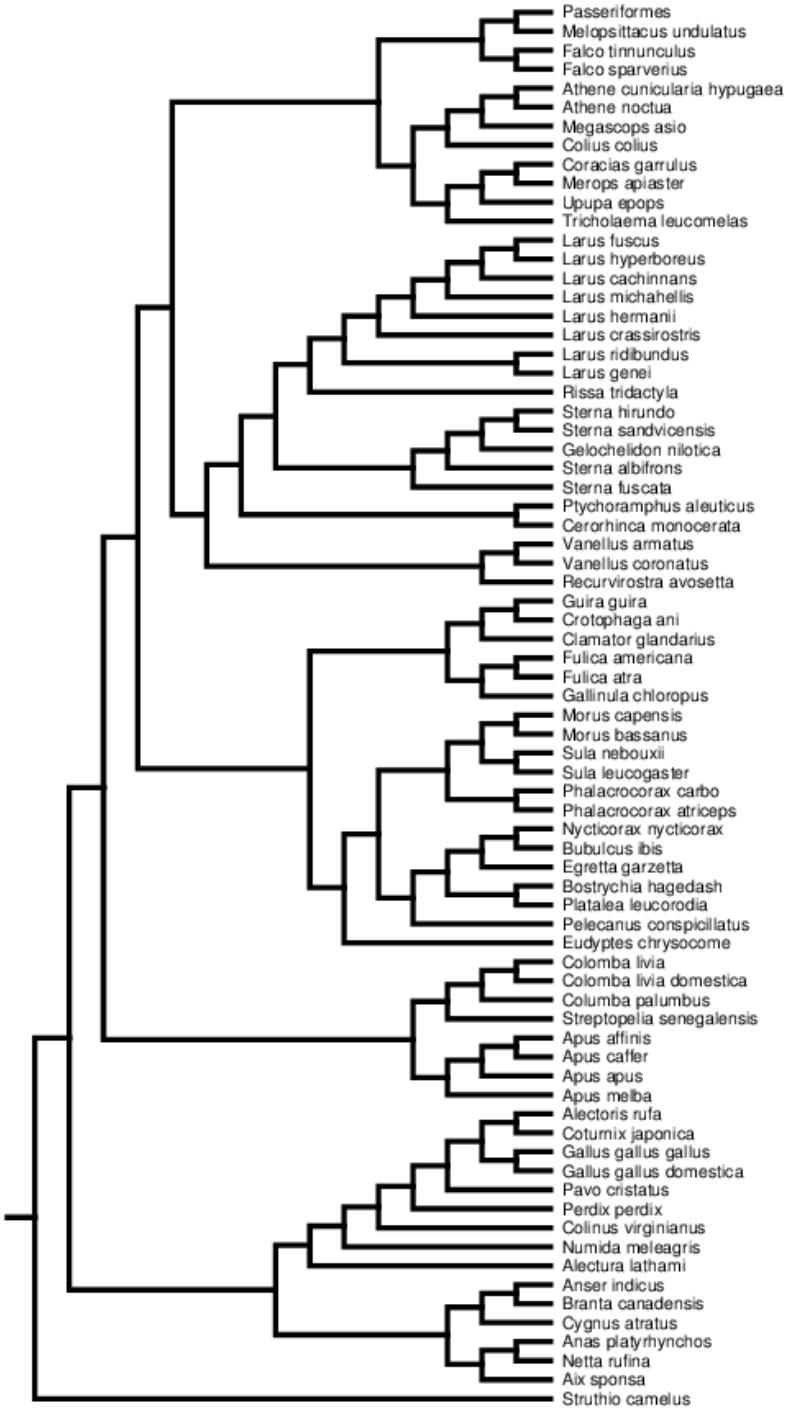


Appendix 2. Topology of the composite phylogeny used in phylogenetic least squares regressions of maternal egg-laying plasma T and species traits.



Appendix 3A. Topology of the passerine portion of composite phylogeny used in phylogenetic least squares regressions of maternal yolk T and species traits.

Appendix 3B. Topology of the non-passerine portion of composite phylogeny used in phylogenetic least squares regressions of maternal yolk T and species traits.



Chapter 3. Within-clutch variation in yolk testosterone as an adaptive maternal effect to modulate avian sibling competition: Evidence from a comparative study

Published in The American Naturalist, 2013, Vol. 181, pages 125-136.

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Abstract

In many species, embryos are exposed to maternal hormones, either in utero, in the egg or in the seed. In birds, mothers deposit substantial testosterone into their eggs, which enhances competitive ability of offspring. These maternal testosterone concentrations vary systematically within clutches in different patterns and may enable mothers to adaptively fine-tune competitive hierarchies within broods. We performed a comparative analysis to investigate this hypothesis using a broad set of avian species. We expected species with small size differences among siblings (arising from small hatching asynchrony or slow growth rates) to aim for survival of the whole brood in good years and therefore compensate late-hatching eggs with relatively more testosterone. We expected species with large size differences among siblings (large hatching asynchrony or fast growth rates) to produce surplus young as insurance against failed offspring and facilitate elimination of redundant surplus young by bestowing late-hatching eggs with relatively less testosterone. As predicted, we found that maternal testosterone compensation to last-hatching eggs is stronger when size differences among siblings become smaller. Maternal testosterone compensation to last-hatching eggs also correlated negatively with hatching asynchrony and growth rates. These findings provide evidence for correlated evolution of several maternal effects that together support different maternal reproductive strategies.

Introduction

Maternal effects, which occur when the phenotype of the mother influences the phenotype of her offspring, were once considered to be little more than nuisance variation in heritability estimates (Wolf and Wade 2009). But since the late 1990s evolutionary ecologists have pushed maternal effects into the spotlight as one of the most important influences on offspring phenotype (Mousseau and Fox 1998, Wolf and Wade 2009). This new interest in the potential adaptive nature of many maternal effects (Marshall and Uller 2007) stimulated substantial theoretical and empirical work addressing whether mothers enhance the reproductive value of individual offspring by matching offspring phenotype to the local environment (Marshall and Uller 2007). Less attention has been paid to the fact that maternal effects can enhance reproductive value at the brood level, by providing an efficient mechanism for mothers to adjust brood size to current food conditions, as food availability is often unpredictable. Throughout the animal and plant kingdom, parents chronically overproduce young, engendering sibling rivalry when young share limited space and resources (e.g. parasitoid wasps, Pexton and Mayhew 2002; damselfly larvae, Anholt 1994; cleistogamous grass, Cheplick 1992; piglets, Fraser and Thompson 1991; see also Mock and Parker 1997). Surplus young can provide extra reproductive value in years when resources are plentiful, or can serve as replacement units when other progeny fail (Mock and Parker 1997). But they become a liability when the number of young exceeds what parents can afford to rear by reducing the per capita share in parental care, which undermines average offspring quality and recruitment prospects.

"Parental favoritism" (Mock and Parker 1997) constitutes a specific class of maternal effects that creates competitive hierarchies among offspring of the same reproductive attempt (such as seeds of the same fruit, eggs of the same clutch, pups of the same litter). These hierarchies channel the bulk of the resources to the more competitive elements of the sibship when resources become scarce, efficiently culling redundant offspring via targeted starvation. Mothers can create such competitive asymmetries by varying propagule size, hormones, cytoplasmic factors, the time at which propagules are released and when they initiate development, and also how fast they develop (Mousseau and Fox 1998, Groothuis et al 2005b). Such mechanisms have been reported in a taxonomically wide range of species (e.g. Ganeshaiah and Uma Shaanker 1988, O'Gara 1969, Fox and Czesak 2000), although whether or not they represent adaptive maternal strategies is rarely tested. Furthermore, evolutionary studies rarely address more than one maternal effect despite the diverse non-genetic mechanisms by which mothers can influence offspring phenotypes that may act simultaneously. Theoretically, multiple maternal effects should function in concert to support a reproductive strategy that will maximize the mother's fitness (Marshall and Uller, 2007). Here, we investigate interspecific correlations among several maternal effects that produce varying degrees of competitive asymmetries within sibships and may adaptively support a maternal reproductive strategy of either culling offspring or promoting survival of all offspring.

One primary avenue by which mothers can produce competitive hierarchies among siblings is by varying propagule size (e.g. echinoderms, Turner and Lawrence 1977; bryozoa, Marshall et al. 2003; iguanas, Castro-Franco et al. 2011; wild radish, Stanton 1984b). Variation in propagule size creates size differences among siblings. Larger propagules produce larger offspring that are more likely to survive (e.g. plants, Black 1958; arthropods, Fox and Csesak 2000; fish, Einum and Fleming 2004; birds, Krist 2011). In many bird species, egg size increases or decreases over the laying sequence within the clutch in relation to their ecology or reproductive strategy (Slagsvold et al.1984).

Mothers can create size advantages in certain offspring also by accelerating their time of emergence from the egg or seed. For example, in plants, mothers can vary seed coat thickness which determines permeability and light exposure and therefore timing of germination (Donohue 2009). Earlier germination confers a size advantage (Stanton 1984a) in sibling competition (Cheplick 1992). In passalid beetles, intermittent oviposition creates hatching asynchrony, giving early-hatching beetles a developmental advantage in lethal sibling combat

(Ento et al. 2010). In birds, mothers commence incubation before clutch completion so that early-laid eggs (laid before start of incubation) hatch together ("core" chicks) followed by the later-hatching eggs (laid during the days that follow the onset of incubation), which produce the asynchronous “marginal” chicks (sensu Mock and Forbes 1995, Mock and Parker 1997). Core chicks grow substantially before the last eggs hatch, so marginal young experience a significant size and developmental disadvantage in the competitive arena and consequently die much more often than core young do (Mock 1984, Magrath 1990).

In addition to varying size of offspring, mothers provision them with variable concentrations of maternal hormones. Maternal hormones influence gene expression and metabolism in seeds (Donohue 2009), can cause diapause in insect eggs (Mousseau and Dingle 1991), determine offspring sex in turtles (Bowden et al 2000), behaviourally lateralize fish (Schaafsma and Groothuis 2012), and affect later stress sensitivity in mammals (Cottrel and Seckl 2009, Weinstock 2008). Their effect on sibling competition has been studied most extensively in birds. The seminal finding of Schwabl (1993) that avian egg yolks contain high concentrations of maternal androgens and that these concentrations increase or decrease over the laying sequence (reviewed in von Engelhardt and Groothuis 2011) opened up the possibility that mothers might adaptively modulate the asymmetries in competitive ability among brood mates via hormonal means (Schwabl 1993, 1996; Groothuis et al. 2005b). More often than not, maternal androgens in avian eggs accelerate pre- and post-natal development rate and result in more competitive behavior in young birds, although they also carry some costs such as compromised immune function and elevated energy expenditure (reviewed in von Engelhardt and Groothuis 2011). The balancing of these benefits and costs to the offspring can therefore yield unique optima for different positions in the laying sequence and may explain the substantial variation in yolk androgen concentrations within clutches. Several studies indicate that genes determine a significant portion of the variation in maternal testosterone transfer to clutches (Gil and Faure 2007; Bertin et al. 2009; Okuliarova et al. 2011) including the pattern in which mothers deposit testosterone in subsequent eggs over the laying sequence (Groothuis et al. 2008). This establishes clear potential for natural selection to shape maternal strategies of differential yolk androgen allocation according to positions of offspring in the brood competitive hierarchy and their corresponding reproductive value.

Birds have proven to be a useful model for studying maternal hormone allocation as well as other maternal effects associated with parental favoritism for several reasons. First of all, the resources and other maternal substances that mothers transfer to eggs are easily measured and manipulated because the egg is relatively large and the embryo develops outside the mother's body. Secondly, competitive hierarchies within broods, individual offspring quality and mortality are easily assessed, both in the lab and in the field. We therefore capitalize on the wealth of data available for avian taxa to investigate whether suites of correlated maternal effects that influence sibling competition might have arisen from long-term evolutionary change driven by the emergence of alternative maternal reproductive strategies that promote survival of the whole brood versus partial reduction of the brood.

The potentially adaptive functions of systematic variation in avian egg size over the laying sequence and size asymmetries within avian broods arising from hatching asynchrony have already been studied using a comparative approach (Slagsvold et al. 1984, Magrath 1990, Amundsen and Slagsvold 1991, Stoleson and Beissinger 1995). To date, however, such a study is lacking for patterns of yolk androgens over the laying sequence. The main adaptive explanation (Schwabl 1993, Lipar et al. 1999) for within-clutch variation in yolk testosterone (T), the most well-studied yolk androgen, proposes that species compensate marginal young with relatively higher maternal T exposure to boost their competitive ability and mitigate the disadvantage hatching asynchrony imposes on marginal young. Therefore, when breeding conditions are favorable, yolk T exposure should enable all marginal young to survive alongside the core and contribute “extra” reproductive value to the brood. However, such species should produce broods containing only small size asymmetries among siblings so that via maternal T compensation, marginal young are able to overcome their initial competitive disadvantage when parents can afford to rear all young. This prediction arises from the fact that past experimental

work shows that the increase in competitive ability due to exposure to elevated yolk T is relatively small compared to the large handicap imposed on marginal chicks by a big delay in hatching (Schwabl 1993, 1996; Eising et al. 2001). Because small size asymmetries among siblings, coupled with an expectation of high maternal T compensation in marginal eggs should help marginal offspring survive, we will refer to these species as "brood survival" species.

Species should not compensate marginal eggs with elevated T if sibling size asymmetries are large because the extra maternal T exposure could not significantly counteract a large size disadvantage. Moreover, marginal young would still be burdened with the costs of elevated maternal T exposure. Species produce large size asymmetries to facilitate adaptive brood reduction of redundant marginal young (Forbes and Mock 2000). These asymmetries might actually be enhanced by giving marginal offspring a lower dose of yolk T compared to the core offspring (Schwabl et al. 1997). Species with large sibling size asymmetries have been suggested to produce marginal young as "insurance" in case a core offspring fails due to stochastic processes such as developmental problems or predation (Forbes 1990, Forbes and Lamey 1996). For example, in obligately siblicidal species, if the core offspring survives, it almost always eliminates the marginal offspring in a fatal fight to the death (Anderson 1990, Mock et al. 1990). The inevitable victory of the core chick is fixed in advance by the mother via an insurmountable size advantage caused by extreme hatching asynchrony (Forbes and Mock 2000). Insurance value for marginal young has also been documented for a wide range of facultative brood reducers, including raptors, parrots and passerines (Wiebe 1996, Forbes et al. 1997). Evolutionary models demonstrate that producing insurance offspring benefits species with large clutches (Forbes 1990), which have a higher cumulative probability of failure of a core offspring. Broods from these species are expected to contain substantial size disparities among siblings despite relatively narrow hatching spreads because growth rates tend to be fast (Ricklefs 1968) which would cause core chicks to attain considerable mass by the time marginal chicks hatch. In species producing marginal young for insurance, brood reduction occurs often, whether it is stochastic mortality of a random nestling, or non-random elimination of a costly redundant marginal offspring (Dorward 1962, Forbes 1990), so we will refer to these species as "brood reducers".

If large size asymmetries and low maternal T compensation are adaptations to promote brood reduction in species producing insurance offspring, and small size asymmetries and high maternal T compensation are adaptations to promote survival of marginal young in good years, then species with small size asymmetries between core and marginal offspring are expected to provide more compensatory maternal T to marginal eggs than are species with large size asymmetries.

In this study, we perform an inter-specific comparative study of within-clutch patterns of yolk T concentrations to investigate whether mothers adaptively allocate yolk T to modulate differences in competitive ability among brood mates. The comparative approach has been successfully applied for testing hypotheses about average clutch levels of yolk androgens (Gorman and Williams 2005, Gil et al. 2007, Schwabl et al. 2007, Martin and Schwabl 2008). To the best of our knowledge, this is the first comparative study on within-clutch variation of these hormones.

First, we tested our prediction that compensatory maternal T for marginal offspring correlates negatively with size disparities within broods. We then investigated how compensatory maternal T correlates with three different factors that cause size disparities within broods. As mentioned above, size asymmetries among siblings develop in species with large hatching asynchrony and in species that have relatively small hatching asynchrony but fast growth rates. In addition, egg size changes linearly over the laying sequence in many species, contributing to some extent to further size variation among siblings, although much less than hatching asynchrony (Slagsvold et al. 1984). We therefore expect compensatory maternal yolk T to correlate with any size disadvantage experienced by marginal young, whether it arises due to a severe delay in hatching, fast growth in core young or small eggs. We tested our prediction that compensatory maternal T correlates negatively with hatching spread and growth rates, and correlates positively with changes in egg size over the laying sequence.

Material and Methods

Life-History variables

We collected data on average hatching spread, clutch size, laying intervals between subsequent eggs within a clutch, logistic growth rate constants, and average egg mass either from databases and handbooks (Cramp 1998, Poole 2005, de Magalhaes and Costa 2009), the primary literature, or our own lab data (doi:10.5061/dryad.b87j7). If estimates for certain parameters were available from several sources, we averaged all values.

Siblings can be divided into two distinct classes of competitive ability: the synchronously hatching "core" part of the brood, and the asynchronously hatching "marginal" part of the brood (Mock and Forbes 1995; Mock and Parker 1997). We calculated the number of core and the number of marginal offspring for each species using information about average clutch size, hatching spread and laying interval between subsequent eggs within a clutch. First we calculated the average number of days mothers require to lay the entire clutch ("laying days"). Hatching asynchrony occurs when mothers initiate incubation before completing the clutch so hatching spread reflects the number of "laying days" during which mothers have already started incubating. The number of asynchronously-hatching, or marginal, eggs can therefore be identified by dividing the hatching spread by the laying interval between subsequent eggs. If these calculations produced non-integers, we rounded the values up. The number of core eggs was calculated by subtracting the number of marginal eggs from the average clutch size.

We collected growth curves from the primary literature and estimated the mass of the core nestlings at the time the last marginal chick hatched based on the average hatching spread for that species. We divided this value by the estimated average mass of a newly hatched chick. This represents the initial proportional mass advantage of core chicks over marginal chicks and is later referred to as "% initial size difference between core and marginal chicks". Growth curves of core offspring are likely to be slightly higher than the mean in species with large asymmetries, which would make our estimation of initial size hierarchies via the mean growth curve conservative.

Within-Clutch Variation in Yolk Testosterone and Egg Size

Information about patterns of yolk testosterone concentrations (doi:10.5061/dryad.b87j7) and changes in egg size (doi:10.5061/dryad.b87j7) over the laying sequence were collected from several sources. We searched the ISI Web of Science database and collected what was available in the primary literature for species with clutches containing more than one egg, until August 2011. We drew from our own unpublished lab data, and contacted colleagues for additional unpublished data.

From these different sources, we extracted mean T concentration values for each egg in the laying sequence, excluding sources that reported only combined measures of several androgens. The most straightforward approach to capture the relevant variation in yolk T was to calculate the difference in average yolk T concentrations between core and marginal eggs (mean marginal T minus mean core T). Yolk T concentrations often do not change over the laying sequence in a linear way, so a comparison of linear slopes of yolk T over the laying sequence, for example, would have introduced substantial noise into the correlation. In our calculations, we included only T concentrations from eggs within the confines of average clutch size and excluded data from additional eggs from clutches of larger size because those values usually came from very small sample sizes and easily introduce biases. If yolk T data came from experimental studies, we used only values from the control group. If studies reported yolk T patterns for both initial clutches and replacement clutches, we included only yolk T from initial clutches in our analyses (doi:10.5061/dryad.b87j7). For species in which we were able to calculate mean yolk T compensation in marginal eggs using data from several studies, we averaged the means from all studies into a single species value. We also compiled mean egg size for consecutive positions in

the laying sequence and calculated the proportional mean difference in size between core and marginal eggs (doi:10.5061/dryad.b87j7) by subtracting mean core egg size from mean marginal egg size and dividing it by mean core egg size.

Our literature search yielded calculations of yolk T compensation for 29 species including the following representation of avian orders: one Anseriformes, five Charadriiformes, one Ciconiformes, two Columbiformes, two Falconiformes, one Gruiformes,13 Passeriformes, three Pelecaniformes, one Psittaciformes. In our statistical analyses, sample sizes differed between models because life history data were not always available for all parameters for all species. Analyses that included hatching spread and growth rate as predictors were performed using a data set containing 25 species. Analyses containing initial within-brood size hierarchy as a predictor, were performed using a data set containing 28 species.

Phylogenetic analyses

We performed all of our ordinary least squares regressions (OLS) and phylogenetic least squares regressions (PGLS) in the R statistical computing environment (R Development Core Team 2012) using the "ape" package (Paradis et al. 2004). The PGLS approach corrects for phylogenetic non-independence of data coming from different species by incorporating a covariance matrix that reflects the degree of their evolutionary relatedness. For this purpose we constructed a composite phylogeny (fig. A1) from several recent molecular bird phylogenies (Crochet et al. 2000, Ericson et al. 2006, Johnsson and Fjeldsa 2006, Hackett et al. 2008). Because we composed a phylogeny from several different published trees, we set all distances between nodes to equal length, which has been demonstrated to be the most conservative approach (Garland et al. 1993, Pagel 1994, Purvis et al. 1994), and branch lengths have been shown not to affect results qualitatively (Poiani and Pagel 1997, Møller et al. 1998, Nunn 1999, Poulin 1999).

We used a PGLS regression that accommodates the degree to which trait evolution depends on phylogeny by estimating the measure of phylogenetic correlation (λ) via likelihood ratio statistics (Freckleton et al. 2002, Hansen and Orzack 2005). A λ of 0 indicates complete phylogenetic independence and produces the same results as a correlation that does not correct for phylogenetic relatedness. A λ of 1 indicates evolution of traits according to the assumption of Brownian motion which expects consistent phenotypic divergence over time due to small random changes accumulating at a constant rate which renders more closely related species more similar. A λ value between 0 and 1 suggests smaller similarity due to common descent than what would be expected under the Brownian model of evolution. In addition to our phylogentically-controlled models in which the PGLS regression estimated λ, we present results from analyses with raw data as suggested by Freckleton et al. (2002) using OLS regressions (in which $\lambda = 0$). We checked each model for non-normality of errors, heteroscedasticity and outliers.

Statistical models

We quantified yolk T compensation as mean T concentrations in core eggs subtracted from mean T concentrations in marginal eggs. Because clutch averages in yolk T concentrations varied widely between species and the biological importance of a certain difference in yolk T compensation between core and marginal eggs is likely to depend on the average absolute T levels within clutches, all models included average core T concentrations as a covariate to scale the level of T compensation to the species average. Including average core T concentrations in our models also corrects for assay differences between studies.

First we performed an ordinary least squares (OLS) regression with yolk T compensation as a response variable that contained the following predictors: 1) the percentage initial size advantage of core chicks over marginal chicks, 2) the proportional difference in size between core and marginal eggs (calculated by dividing the mean difference in size between core and marginal eggs by mean core egg size), and 3) average core T concentrations. The first predictor combined the contributions of hatching spread and growth rates to initial size

asymmetries. The second predictor adds the contribution of within-clutch egg size variation to initial size asymmetries. We included the third predictor to correct for the species' average clutch T concentrations. We repeated this model using a PGLS regression that corrected for phylogenetic relatedness.

Second, we performed an OLS regression with yolk T compensation as a response variable with predictors that represent the three main contributing factors to initial size asymmetries: hatching asynchrony (predictor 1), growth rate (predictor 3), and the differences in size between core and marginal eggs (predictor 5) plus several additional covariates (predictors 2, 4 and 6) that we included to correct predictors 1, 3 and 5. In these models, the predictors therefore included 1) average hatching spread, 2) the number of marginal eggs (to correct hatching spread for the number of marginal eggs hatching during the time interval), 3) logistic growth rate constant, 4) mean core egg size (to correct for the fact that growth rate depends to some extent on body size), 5) difference in size between core and marginal eggs (marginal egg size minus core egg size), and 6) mean core T concentrations. We repeated this model in a PGLS regression that corrected for phylogenetic relatedness.

Each model represented a specific hypothesis about the contributions of groups of factors to initial size asymmetries that might have driven the evolution of different patterns of yolk testosterone over the laying sequence. Some predictors were included as covariates to correct other data (e.g. in the second model: the number of marginal eggs, mean core egg size, mean core T concentrations). As all predictors were important for properly testing our hypotheses, we perform no model simplification. The PGLS package we used in R did not produce residuals corrected for phylogeny so we present phylogentically uncorrected residuals in figures. All of our analyses were two-tailed with a 95% confidence level.

Results

Initial size asymmetries within broods

As predicted, in our raw species data analysis using an OLS regression ($n = 28$), in species in which core young have a relatively small initial size advantage over their marginal siblings, mothers provide relatively more compensatory yolk T to marginal eggs compared to species in which core young have a relatively large size advantage over their marginal siblings ($b = -0.76$, s.e. = 0.19, $p = 0.001$; fig. 1). Maternal yolk T compensation did not vary with the degree to which egg size increased or decreased over the laying sequence ($b = 1.20$, s.e. = 2.73, $p = 0.665$), or with average yolk T concentrations in core eggs ($b = 0.16$, s.e. = 0.09, $p = 0.101$).

We then performed the same regression on the same species, but controlled for phylogenetic relatedness (PGLS regression), and found qualitatively the same results: species in which core young have a relatively small initial size advantage over their marginal siblings, mothers provide relatively more compensatory yolk T to marginal eggs compared to species in which core young have a relatively large size advantage over their marginal siblings ($b = -0.70$, s.e. = 0.20, $p = 0.002$). Again, maternal yolk T compensation did not vary with the degree to which egg size increased or decreased over the laying sequence ($b = -0.93$, s.e. = 2.93, $p = 0.754$) or with average yolk T concentrations in core eggs ($b = 0.13$, s.e. = 0.09, $p = 0.168$). This model estimated λ at 0.31, indicating some dependence on phylogeny.

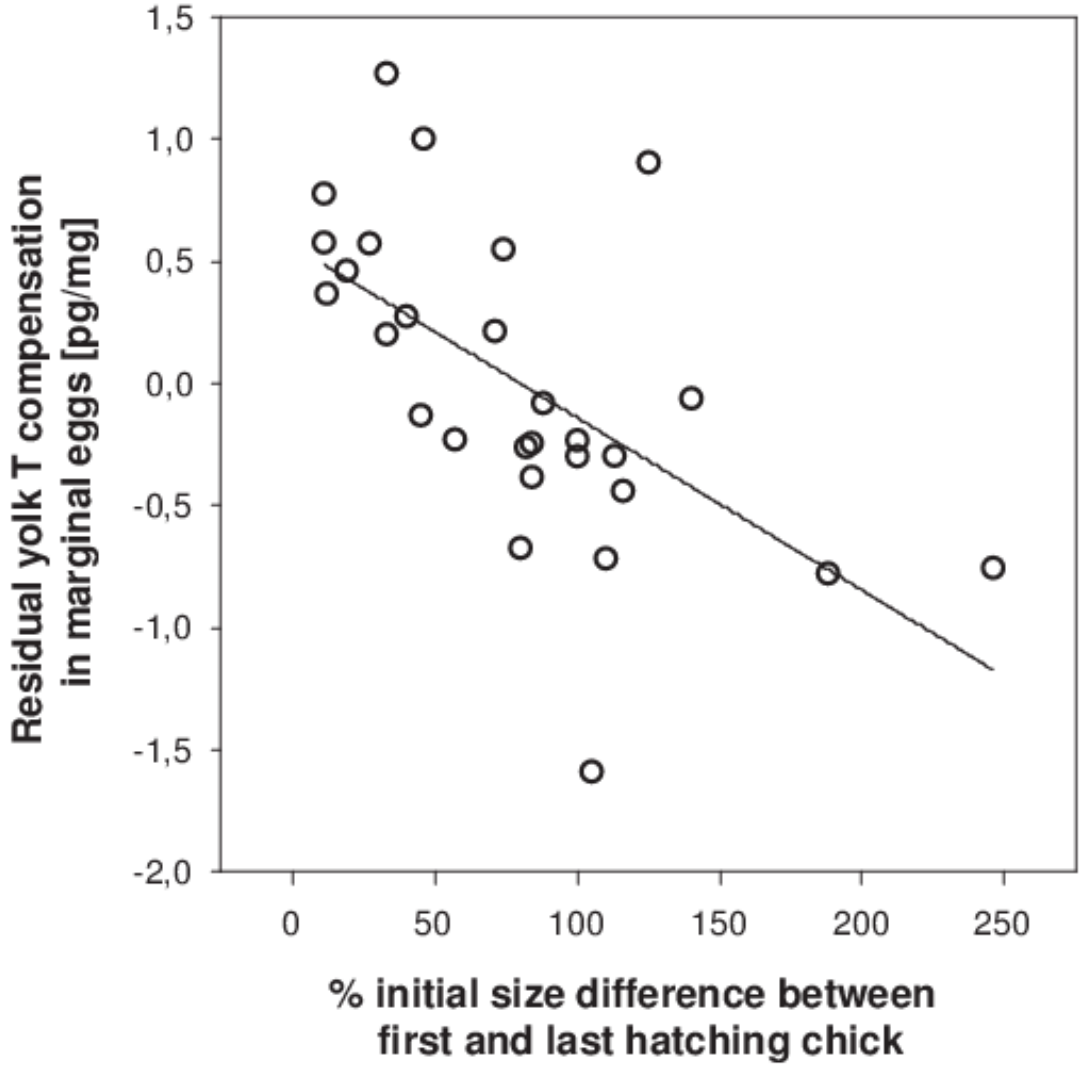


Figure 1: Maternal compensatory yolk T in marginal eggs, controlled for mean core yolk T concentrations and proportional size difference among core and marginal eggs, in relation to percentage initial size advantage of core chick over marginal chick (n=28). Yolk T compensation calculated as mean T concentrations in core eggs subtracted from mean T concentrations in marginal eggs. % initial size difference between first and last hatching chick calculated from species growth curves as mass of the core nestlings by the time the last marginal chick hatched (timing estimated from hatching spread), divided by the mass of the marginal chick.

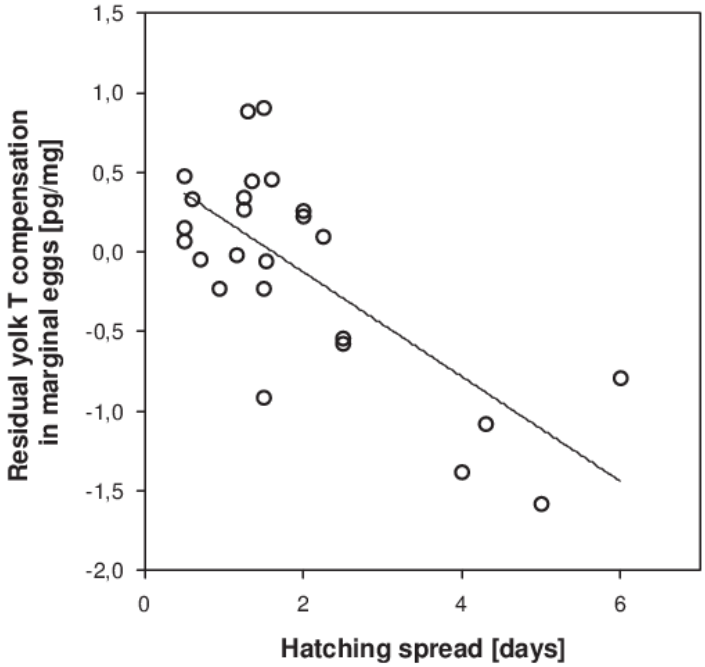


Figure 2: Maternal compensatory yolk T in marginal eggs, controlled for number of marginal eggs, growth rate, mean core T concentrations, mean egg size, and difference in size between core and marginal eggs, in relation to hatching spread (n=25). Yolk T compensation calculated as mean T concentrations in core eggs subtracted from mean T concentrations in marginal eggs.

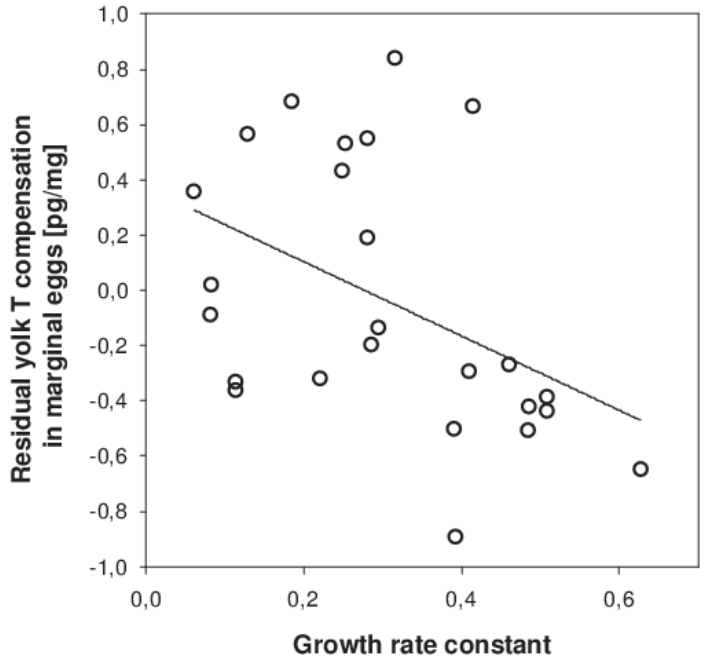


Figure 3: Maternal compensatory yolk T in marginal eggs, controlled for number of marginal eggs, hatching spread, mean core T concentrations, mean egg size, and difference in size between core and marginal eggs, in relation to logistic growth rate constant (n=25). Yolk T compensation calculated as mean T concentrations in core eggs subtracted from mean T concentrations in marginal eggs.

Hatching spread, growth rate, and change in egg size

As predicted, when using raw species data in an OLS regression (n = 25), in species with small hatching asynchrony, mothers provide relatively more compensatory yolk T to marginal eggs compared to species with large hatching asynchrony b = -0.43, s.e. = 0.09, p <0.001; fig. 2). In the same model, we found that in species with slow growth rates, mothers also provide relatively more compensatory yolk T to marginal eggs compared to species with fast growth rates (b = -3.57, s.e. = 0.99, p = 0.002; fig. 3). In the same model, maternal yolk T compensation did not vary with the degree to which egg size increased or decreased over the laying sequence (b = 0.09, s.e. = 0.12, p = 0.476), average core egg size (b = -0.01, s.e. = 0.005, p = 0.076), or with the number of marginal eggs (b = 0.11, s.e. = 0.10, p = 0.249). In the same model, we found that species that produced eggs with higher average core yolk T concentrations also provided marginal eggs with relatively more compensatory yolk T (b = 0.17, s.e. = 0.08, p = 0.046) compared to species with lower average core yolk T concentrations.

When we performed the same regression on the same species, but controlled for phylogenetic relatedness by allowing the regression to estimate λ, we found that again mothers provide relatively more compensatory yolk T to marginal eggs compared to species with large hatching asynchrony (b = -0.35, s.e. = 0.10, p = 0.002). We also found that in species with slow growth rates, mothers provide relatively more compensatory yolk T to marginal eggs compared to species with fast growth rates (b = -3.82, s.e. = 1.13, p = 0.003). In this model, mean core egg size (b = -0.01, s.e. = 0.005, p = 0.087), the degree to which egg size increased or decreased over the laying sequence (b = 0.11, s.e. = 0.10, p = 0.291), the number of marginal eggs (b = 0.06, s.e. = 0.10, p = 0.548) and average core yolk T concentrations (b = 0.10, s.e. = p = 0.196) did not vary significantly with the degree to which mothers provided compensatory yolk T to marginal eggs. In this latter model, λ = 0.69, indicating moderate phylogenetic dependence in the correlations.

Discussion

In a wide variety of plant and animal species maternal effects can have important consequences for individual offspring, but their combined effect on a set of propagules that comprise the same reproductive attempt has hardly been studied (see introduction). In addition, maternal effects encompass a wide array of pathways, potentially providing the mother with a diverse tool kit for optimizing her reproductive performance in different environmental conditions, and are expected to have evolved to work in concert. Understanding their interplay might therefore be crucial for unlocking the importance and evolution of maternal effects. We performed the first phylogenetic comparative study exploring correlations among several maternal effects across a broad array of avian species that encompass long periods of evolutionary change in diverse environments. As in many other taxa, avian mothers expose their embryos to substantial amounts of maternal androgens by depositing testosterone in their eggs, affecting behavior and physiology of the chicks (von Engelhardt and Groothuis 2011). As avian mothers differentially deposit testosterone according to the position of the egg in the laying order of the clutch, these maternal hormones affect the chicks' competitive positions in the sibling hierarchy, potentially affecting the reproductive value of the brood as a whole (Schwabl 1993, Groothuis et al 2005). Our aim was to test the prediction that within-clutch patterns of maternal androgens evolved together with maternal effects that cause size asymmetries within broods, (egg size, hatching asynchrony, growth rate) to support maternal reproductive strategies of brood survival or brood reduction. Our study focused on the parental favoritism hypothesis which postulates that mothers confer competitive advantages and handicaps on different individuals of their broods to create competitive hierarchies. These hierarchies facilitate a secondary adjustment of brood size if the number of young exceeds what parents can afford to rear (Mock and Parker 1997).

We found strong evidence that last-hatching eggs ("marginal" eggs) from species in which siblings are relatively similar in age and size (“brood survival” species), receive more maternal T relative to first-hatching eggs ("core" eggs), compared to species in which age and

size differs substantially between siblings ("brood reducing" species, fig. 1). This suggests that mothers in species that produce marginal young primarily intended to provide a reproductive bonus in good years, compensate their disadvantage in the hatching order and sibling rivalry with relatively more yolk T compared to species producing marginal young mostly for replacement value. In the latter case marginal chicks should only survive in case the core chicks did not hatch or died early in life.

To further explore how maternal androgens relate to the specific processes that give rise to size asymmetries among siblings, we tested the correlation of yolk T compensation with three contributing factors to within-brood variation in age and size: hatching spread, growth rate and difference in size between core and marginal eggs. We found no relationship between yolk T compensation and the egg size differences in any of our models. The lack of a correlation between these factors probably comes from the fact that variation in egg size contributes only little to within-brood size asymmetries compared to the combined contributions of hatching spread and growth rate. In our dataset, the maximum difference in size between core and marginal eggs was only 7.5 % (*Sula leucogaster*, Ceyca and Mellink 2009), while in 10 out of the 28 species we analyzed, the first chick grows to be at least 100% larger than the last chick by the time all chicks have hatched. Clearly, most of the variation in age and size among brood mates arises due to hatching asynchrony (Parker, 1982) and due to the growth of the early-hatching core part of the brood during the time lag before the marginal young hatch. In line with our predictions, we found that species with high compensatory maternal T in marginal eggs had a relatively small degree of hatching asynchrony (fig. 2) and slow growth rates (fig. 3) compared to species with low compensatory maternal T in marginal eggs. Species with large hatching spreads are known to employ an insurance strategy (Forbes and Mock 2000). Species with fast growth rates are also expected to employ an insurance strategy due to their tendencies to produce large clutches (Ricklefs 1968), a trait which favors the production of insurance offspring (Forbes 1990). We show that both of these traits are associated with very low maternal T compensation.

David Lack's (1947, 1954, 1968) resource-tracking hypothesis proposes that many avian species produce optimistic clutch sizes so they can capitalize in years when food is abundant but use hatching asynchrony to designate marginal chicks as expendable for when food becomes too scarce for the parents to rear the whole brood. Marginal chicks therefore contribute "extra" reproductive value to the brood by surviving alongside the core under favorable conditions (increasing the quantity of young). But they don't compromise the value of the core under poor conditions (Forbes et al 2002) because they compete ineffectively when food is very limited and die of starvation (preserving quality of young). We suggest that these species should produce broods with narrower size hierarchies and more yolk T compensation in marginal eggs. This combination of traits would enhance the survival of marginal young in years when resources are plentiful because food returns would more than compensate for the extra begging effort and growth (reviewed in von Engelhardt and Groothuis 2011) and allow them to overcome their initial small size disadvantage.

In poor years, however, yolk T might actually accelerate mortality in marginal offspring because they receive insufficient food returns to compensate for their higher energy requirements (as suggested in Tobler et al 2007). Considering that the behavioral and physiological downstream effects of yolk T exposure place a substantial burden on a chick's energy budget (Tobler et al 2007), and perhaps therefore also on its investment in immunity (Groothuis et al 2005a), such context-dependent effects like in good vs. poor food conditions are very likely to occur, although they have not yet been directly tested. The fact that yolk T compensation might benefit marginal young in good conditions and harm them in poor conditions has already been suggested by Royle et al (2001) and Groothuis et al (2005a). If this is the case, then as we showed in our correlations, the insurance species, which have large sibling size hierarchies, should not compensate marginal eggs with yolk T. In these species, redundant marginal young inevitably experience "poor conditions" and yolk T compensation would risk accelerating brood reduction when ineffective high begging effort causes them to perish prematurely before the core is out of the high risk period of failing.

Here, we have suggested that the patterns of yolk T compensation, as correlated with within-brood size asymmetries, support contrasting reproductive strategies in which mothers produce marginal chicks for "extra" reproductive value vs. "insurance" value. For the former strategy mothers produce broods with narrow hatching spreads and substantial yolk T compensation in marginal eggs so that marginal young have a chance to survive in years with abundant food. For the latter strategy, mothers produce broods with large hatching spreads and little yolk T compensation to facilitate the elimination of redundant marginal young when they are not needed to replace failed core offspring. This model rests on the assumption that elevated yolk T exposure benefits marginal young in good breeding conditions but harms them in poor breeding conditions. Inconsistencies in the outcomes of experimental studies that manipulated yolk T suggest that this is likely to be the case but it needs to be tested systematically within one experiment. But context-dependency of maternal effects has already been demonstrated convincingly as a broad phenomenon occurring in diverse taxa (e.g. frogs: Kaplan 1992, trout: Einum and Flemming 1999, beetles: Fox 2000, soil mites: Plaistow et al 2006).

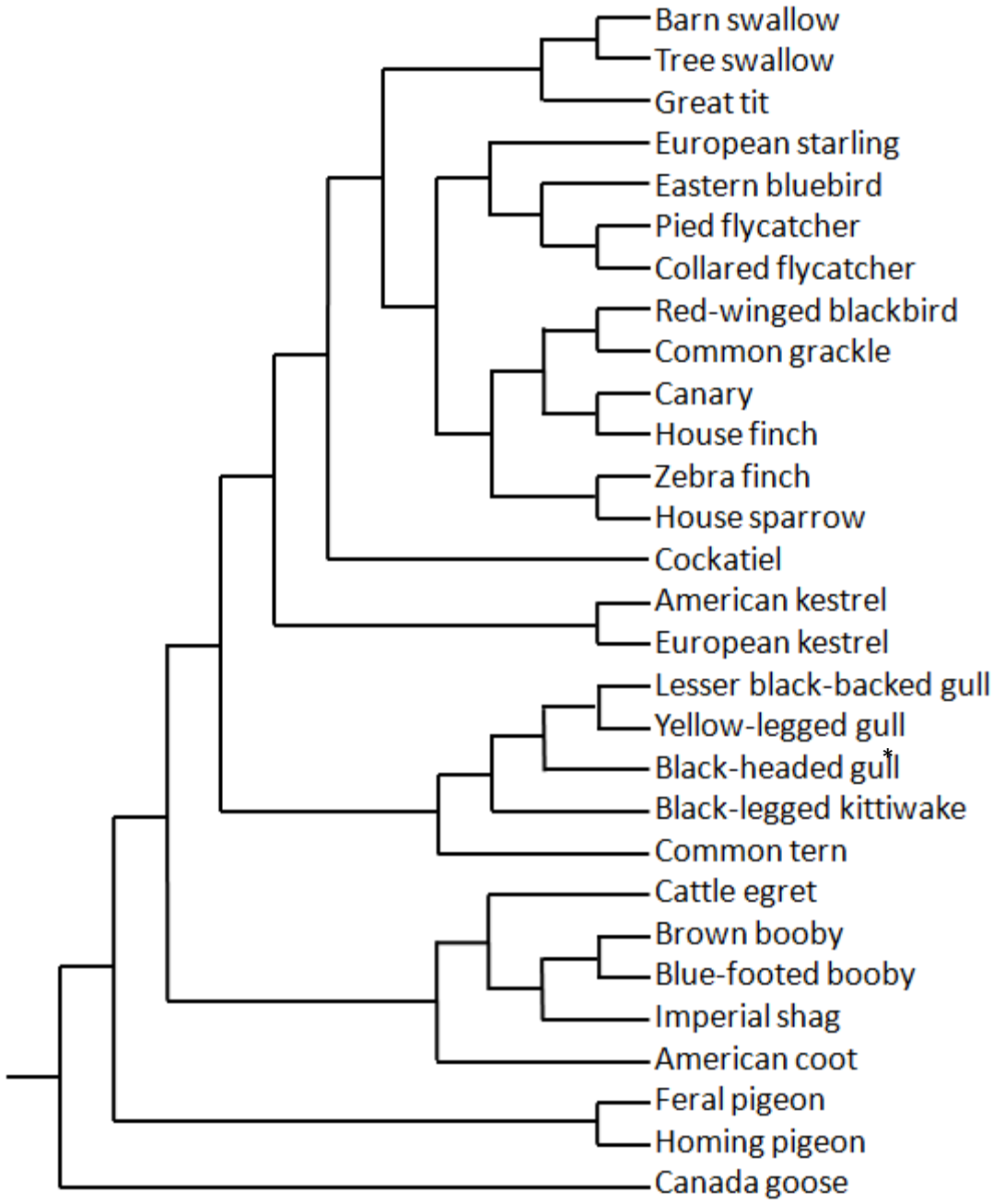
In our analyses, we averaged the patterns of yolk T over the laying sequence for each species and assume it to be the product of evolutionary pressures related to competitive hierarchies among siblings. We find the strong relationships that we expected, which supports this assumption, but variation among species averages may not be the whole story. Substantial variation in yolk T patterns over the laying sequence exists also within species, and several studies have shown that this variation correlates with characteristics of the mother or to aspects of her environment like food abundance (Rutstein et al 2004, Sandell et al 2007, reviewed in von Engelhardt and Groothuis 2011). If species are flexible to adjust patterns of yolk T over the laying sequence according to breeding conditions, then mothers could provide marginal young with more compensatory yolk T to promote their survival in good breeding conditions (e.g. Vergauwen et al 2012). With such flexibility, mothers could also provide marginal young with less compensatory yolk T in anticipation of poor conditions and a high likelihood of brood reduction ("hatching asynchrony adjustment hypothesis", Groothuis et al 2005b). It is also known that the hatching spreads can vary substantially within species, and mothers have been shown to produce more asynchronous broods in anticipation of difficult rearing conditions (e.g. Wiebe and Bortolotti 1994). If mothers are able to adjust hatching spreads and yolk T compensation in concert and in response to environmental conditions as indicated for the black-headed gull (Muller et al 2004) then we might expect to find the negative correlation between those two factors that we observe in a between-species comparison, also in a within-species comparison.

In this study we have investigated the correlated evolution of a suite of maternal effects that appear to function together to allow mothers to optimize the number of progeny that they rear in a given breeding attempt. We focused on variation in maternal androgen exposure among siblings, variation in propagule size among siblings, and variation in relative timing of emergence from the egg. Other studies suggest that similar processes may have evolved in other taxa as well. For example, studies on montane lizards show complex interactions between maternal hormones, egg size and sex on prenatal development rates causing variation in hatching times and thus conferring developmental advantages on select offspring (Radder and Shine 2007). Subsocial bugs covary egg size and spatial position within the clutch so that peripheral (insurance) eggs which are exposed to higher predation risk are smaller (Kudo 2006). In plants, the potential for maternal effects in enhancing growth rates, accelerating emergence or increasing seed weight as an avenue toward conferring an early size advantage to seedlings has been implied (e.g. Stanton 1984b) but not directly studied in the context of sibling rivalry. We suggest that the hypothesis we tested in this study extends to all taxa with sibling rivalry: mothers which anticipate a high likelihood of brood reduction should create competitive asymmetries among young supporting the best quality offspring. But, mothers that aim for survival of all offspring should try to equalize the competitive abilities of siblings so that no offspring are outcompeted.

Acknowledgments

First of all we would like to thank Giuseppe Boncoraglio for helping us to collect a portion of the life history data from databases, yolk T data from the literature and participating in preliminary analyses. We gratefully thank V. Garcia-Fernandez, D. Gil, G. Hegyi et al., W. Műller, K. Navara, A. Raven, E. van der Steen, R. Van Noordwijk, and N. Von Engelhardt, for providing us with unpublished data. We thank Ido Pen and Lazlo Garmaszegi for statistical advice, Jan Komdeur for providing us with helpful comments on our manuscript, the editor Judith. L Bronstein and two anonymous reviewers whose feedback improved the manuscript.

Appendix A. Topology of composite bird phylogeny used in comparative analyses.



Chapter 4. Maternal androgens increase sibling aggression, dominance, and competitive ability in the siblicidal black-legged kittiwake (*Rissa tridactyla*)

Published in PLoS ONE, 2012, 7: e47763

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Abstract

Animals and plants routinely produce more offspring than they can afford to rear. Mothers can favour certain young by conferring on them competitive advantages such as a leading position in the birth sequence, more resources or hormones. Avian mothers create hatching asynchrony within a clutch and at the same time bestow the eggs with different concentrations of androgens that may enhance or counteract the competitive advantage experienced by early-hatching "core" young. In siblicidal birds, core young assume a dominant social position in the nest due to their size advantage and when threatened with starvation fatally attack subdominant later-hatching "marginal" young. A role for maternal androgens in siblicidal aggression has frequently been suggested but never tested. We studied this in the facultatively siblicidal black-headed kittiwake. We found that marginal eggs contain higher instead of lower concentrations of androgens than core eggs. Surprisingly, exposure to experimentally elevated yolk androgens increased sibling aggression and dominance, even though in nature marginal eggs never produce dominant chicks. We propose the "adoption facilitation hypothesis" to explain this paradox. This cliff-nesting colonial species has a high adoption rate: ejected marginal kittiwake chicks frequently fall into other nests containing chicks of similar or smaller size and exposure to yolk androgens might help them integrate themselves into a foster nest.

Introduction

With each reproductive attempt, organisms face the critical decision of how many progeny to produce. According to life history theory, their choices reflect an optimized trade-off between offspring quality and offspring quantity (Lack 1947, Smith and Fretwell 1974, Einum and Fleming 2000) as well as contributions of current versus future reproductive success to parental fitness (Williams 1966a, 1966b). These trade-offs arise due to the costs of producing offspring and in many species, progeny number becomes limited by substantial investment into costly parental care during the early postnatal life of offspring (Mock and Parker 1997). The capacity of parents to deliver the requisite care to young depends heavily on resource availability during the reproductive season, which they are often unable to predict accurately (Lack 1947, 1954). Animals as well as plants chronically overproduce offspring but appear to have evolved mechanisms to adjust their broods secondarily to a more modest number when the number of viable progeny exceeds what parents can afford to rear (Mock and Parker 1998). Mothers can either cull their brood directly via cannibalism (e.g. rodents: Schneider and Wade 1991, amphipods: Sheader 1983, fish: Fitzgerald and Whoriskey 1992) or they can facilitate sibling rivalry-mediated brood reduction by conferring phenotypic handicaps or advantages on certain young (e.g. pronghorn embryos: O'Gara 1969, seeds of *Dalbergia sissoo*: Gasneshaiah and Uma Shaankar 1988, canary eggs: Schwabl 1993) so that competitively superior siblings can eliminate competitively inferior siblings when resources are scarce (Glassey and Forbes 2002).

The role of maternal effects in modulating fatal sibling rivalry has been studied most extensively in birds, perhaps in part due to the conspicuous disadvantage imposed by many avian mothers on parts of their broods via hatching asynchrony (Magrath 1990). Hatching asynchrony occurs when mothers begin incubating eggs before they finish laying the entire clutch which initiates early onset of development in the already-laid eggs (core eggs, *sensu* Mock and Forbes 1995, Mock and Parker 1997) and results in delayed development and hatching in later-laid eggs (marginal eggs, *sensu* Mock and Forbes 1995, Mock and Parker 1997). Asynchronous hatching causes chicks from marginal eggs (marginal chicks) to be smaller and competitively inferior and therefore to have much lower survival than their core siblings (Mock 1984, Magrath 1990). In many avian species, marginal young die of starvation under poor food conditions when they repeatedly fail to receive food delivered by parents because of their relatively smaller size and retarded motor development (scramble competition, Glassey and Forbes 2002). Siblicidal species constitute a special case in which age and size asymmetries within broods translate into clear dominance hierarchies, and subordinate marginal young die primarily because dominant core siblings attack them, resulting in fatal wounds or exile from the nest (Drummond 2006, Mock and Parker 1997).

While within-brood size asymmetries are caused predominantly by hatching asynchrony (Glassey and Forbes 2002), mothers may further fine-tune competitive asymmetries by allocating more androgens to certain eggs. Avian mothers deposit high concentrations of androgens into egg yolks that vary in systematic patterns over the laying sequence (for reviews see Groothuis et al 2005, Gil 2008, Von Engelhardt and Groothuis 2011). Exposure to elevated yolk androgens often promotes faster pre- and post-natal development in young, more begging to parents for food and increased territorial aggression (reviewed in Groothuis et al 2005, Gil 2008, von Engelhardt and Groothuis 2011). Increasing and decreasing patterns of yolk androgen allocation over the laying sequence might be an adaptation to promote brood survival and brood reduction strategies respectively by compensating or enhancing within-brood size asymmetries caused by asynchronous hatching (Schwabl 1993, 1996; Groothuis et al 2005).

However, the function of yolk androgen exposure in the context of sibling competition has only been studied in species in which brood reduction occurs via scramble competition for food (reviewed in von Engelhardt and Groothuis, 2011), and has never been studied in a species in which broods are reduced by core siblings using overt aggressive behaviour against their marginal siblings. Following the publication of a very frequently-cited study reporting that total yolk androgens decrease over the laying sequence in the facultatively siblicidal cattle egret (Schwabl et al 1997), numerous papers implied that relatively higher maternal androgens in

core eggs might aid siblicidal brood reduction of marginal young by increasing aggression and/or dominance in the core young. although since then, variable patterns of yolk androgen deposition have been reported for other siblicidal species: yolk androgens increase over the laying sequence in black-legged kittiwake (Vallarino et al 2012), in Australian pelicans (G. Johnston, unpublished data), and in blue-footed boobies in poor breeding conditions (Dentressangle et al. 2008); yolk androgens show no significant change over the laying sequence in brown and blue-footed boobies (Drummond et al. 2008); and yolk androgens decrease over the laying sequence in blue-footed boobies in good breeding conditions (Dentressangle et al. 2008). Yolk androgen exposure increases territorial aggression directed toward unrelated young in non-siblicidal gulls, but not to siblings (Müller et al 2009), and its effects on siblicidal aggression in siblicidal species are still not known.

We investigated the function of within-clutch variation of yolk androgens for sibling competition and brood reduction in the facultatively siblicidal black-legged kittiwake (*Rissa tridactyla*), which lays a modal clutch size of two eggs (Cullen 1957). When food is scarce, the first-hatching core chick eliminates the second-hatching marginal chick via aggressive attacks that cause the marginal chick to fall out of the nest (Braun and Hunt 1983). But in years with sufficient food availability, core chicks tolerate the presence of the marginal chick, although a clear dominance hierarchy exists in which the subdominant chicks regularly performs submissive displays (Braun and Hunt 1983, White et al 2010).

In our study, we measured yolk androgen concentrations from first- and second-laid eggs to investigate whether yolk androgens increase over the laying sequence in our kittiwake population. Then we manipulated androgen concentrations in kittiwake eggs and created artificial sibling pairs matched for age and weight, containing one chick exposed to a high androgen level and one chick exposed to a lower yolk androgen level to determine whether exposure to yolk androgens enhances competitive ability in chicks and might therefore provide a mechanism for mothers to adaptively fine-tune competitive hierarchies within broods. If yolk androgens increase over the laying sequence, we hypothesize that mothers intend yolk androgens to compensate marginal young and should therefore enhance begging behaviour, speed up growth and development, but have no influence on aggression. If yolk androgens decrease over the laying sequence, we hypothesize that yolk androgens enhance aggressive behaviour towards the sibling so that mothers facilitate brood reduction by conferring a competitive advantage on core young via increased deposition of maternal androgens in the first egg.

Methods

Study colony and species

All eggs used in this study came from a large, dense black-legged kittiwake colony on the Ekkerøy peninsula in Norway. Approximately 20,000 pairs of kittiwakes breed on the south-oriented cliff that rises steeply 40-50 meters from the Varanger fjord and stretches ca. 1 kilometer along the coast. Kittiwakes lay a modal clutch size of two eggs (Hatch et al. 2009) with an average laying interval of 2.5 days between eggs (Hatch et al. 2009). In this species, the second chick hatches ca. 1.3 days after the first egg (Hatch et al. 2009, Braun and Hunt 1983).

Ethics statement

Egg collection was licensed by the Directoratet for Naturforvaltning in Tromsø, Norway. The experiment was executed according to a protocol approved by the Animal Experimentation Committee of the University of Groningen under license DEC 3543.

In the evenings, chicks from dyads that showed aggression were separated in cages by a barrier. In the cages in which we hadn't observed aggression, we placed small wooden nest boxes into the cages so that submissive chicks could find refuge in case the dominant chick became aggressive.

Kittiwake chicks show two types of aggressive behaviour: low and high intensity aggression. Low intensity pecks serve to enforce a dominance hierarchy and elicit submissive postures from the subdominant sibling. Often dominant chicks lunge towards their sibling but their attempts to peck do not even result in contact with the sibling and represent more of a threat display. The second type of aggression is the escalated violence that precedes siblicide and is comprised of frequent intensive pecks. Over the short term this does not injure chicks although subdominant chicks move away to escape attacks. In kittiwake colonies, chicks occupy relatively small nests built on the sides of cliffs and siblicide occurs when aggressive dominant chicks attack subdominant chicks and push chicks out of the nest. Chicks lack the weaponry and strength to inflict serious injuries on their siblings. In our experiment, the aggression we observed were primarily the dominance pecks, and we only rarely observed escalated aggression. In the few cases in which aggression escalated to the intensity that precedes siblicide in a natural setting, chicks were immediately separated. We recorded presence/absence of aggression only before meals (ca. every 5 hours) to increase statistical independence of consecutive records of aggression. But we monitored chicks more frequently (ca. every 2 hours). Only occasionally did chicks begin to show intense aggression already well before meal times and in those cases they were separated.

A few chicks died from developmental problems at hatching and a few chicks died from unknown causes (we suspect a nutrient lacking from their diet). We undertook several actions to counteract the latter by contacting several experts and trying to optimize food with daily fresh fish for example (as this is their natural diet).

Most chicks were euthanized, but 6 chicks were raised in outdoor aviaries to adulthood, so that we could fine-tune the composition of their diet for future experiments with this species. After 4 months in the outdoor aviaries, these birds were also euthanized. Euthanizing the birds was obligatory and forced on us by the governmental inspector for ethical issues in animal experiments. We euthanized the chicks via lethal Pentobarbital injection as specified by animal welfare guidelines at our university.

Egg collection, hormone measurement, and injection

In May 2009, we collected 14 freshly-laid eggs (seven first eggs, seven second eggs, all coming from 7 clutches) from the kittiwake colony in Ekkerøy. Empty nests were marked and checked every day for new eggs. After eggs were collected, yolks were immediately separated from albumin and stored at -20 degrees C. Yolks were later thawed, weighed, and diluted in 1 mL filtered, deionized water (Milli-Q) per gram of yolk. We extracted testosterone and androstenedione from approximately 300 mg of diluted yolk sample using an extraction and radioimmunoassay protocol described in Schwabl (1993).

In mid-May 2010, we monitored 176 nests in the Ekkerøy colony daily and collected freshly-laid first eggs only. Eggs were stored at ca. 12 degrees C for 1-5 days (below physiological zero) and then were transported back to the Netherlands and injected the following day. We injected 40 eggs with 50 µl of vehicle (sterile sesame oil) and 40 eggs with 50 µl of androgen solution of the same oil containing 0.153 µg testosterone and 2.695 µg androstenedione (Sigma, Germany). This amount of androgen represents the average difference in total testosterone and androstenedione between first and second-laid black-legged kittiwake eggs (testosterone levels from Vallarino et al. 2012, androstenedione from unpublished data from D. Gil, mean yolk size: 12.92g, standard error: 0.215g from Vallarino 2008). Our androgen injections elevated androgen concentrations in eggs to a mean level that fell within one standard error of the mean concentrations we measured in second-laid eggs from our population (see results and Fig. 1A and 1B) We injected laterally-held eggs from an off-center point on the shell and inserted the needle at a 45 degree angle so that it would penetrate the yolk without damaging the blastocyst. Injections were made with a 25 G needle and the hole in the shell was closed with a tiny square of artificial skin (Hansaplast). We then placed all eggs into one incubator at 37.5 degrees C and 50% humidity until they hatched (ca. 26 days).

Experimental design

After all chicks hatched, we created artificial sibling dyads of age- and size-matched androgen and control chicks (mean age at pairing 29.5 hours, ranging from 0 to 51 hours old) and housed each dyad in individual cages with constant light mimicking the 24 hours of daylight they would experience in an arctic Norwegian summer. The experiment room was lit with fluorescent bulbs that meet the standards for animal housing facilities in the Netherlands. In addition, each cage was lit with a 25-Watt bulb during the first week of the experiment to provide warmth for very young chicks. Light intensity did not change over the course of the day. Chicks were fed a mixture of frozen cod and fresh European anchovy and smelt 5 times per day, and on day 20 of the experiment we began supplementing their diet with rehydrated trout pellets. Chicks remained in the same dyad for several days at a time (mean=7.04 days, s.e. = 0.67) and then were swapped into new dyads because each new sibling combination presented a new opportunity to test the effect of yolk androgen exposure on dominance . We created 52 unique chick dyads in total (number of chicks experiencing at least one dyad: n = 39; at least 2 dyads: n = 32, at least 3 dyads: n = 20, at least 4 dyads: n = 12; 5 dyads: n = 1). Because several times during the experiment androgen chicks outnumbered control chicks, we occasionally created sibling dyads containing two androgen chicks. Even though those dyads did not allow us to test effects of elevated androgen exposure, we were able to include them in analyses investigating winner-loser effects of dominance status across consecutive dyads. The experiment lasted 28 days.

Growth and development

After hatching began, incubators were checked every 3 hours. We recorded hatching time and hatching success. We measured body mass and bill length of all chicks at hatching and at 10 additional time points thereafter (days 0, 2, 4, 6, 8, 14, 16, 18, 20 and 25 of the experiment). Bill length correlates linearly with tarsus in kittiwake chicks (Müller, unpublished data), indicating that it is a good measure of structural body size, but it has higher repeatability than tarsus measurements. We also recorded age and treatment of any deaths that occurred during the experiment.

Behaviour

We scan-sampled aggressive and submissive behaviour in all dyads at sampling points spaced several hours apart (n = 48 sampling points, range in intervals between sampling: 1.9 – 74 hrs, median = 7 hrs). After entering the experiment room we noted which chicks were showing aggression toward their sibling, via aggressive pecks on the body, neck and head of the victim, and which chicks were showing submissive behaviour characterized by tucking the bill under the body and exposing the black band of down across the nape (Cullen, 1957). We also recorded whether chicks performed begging vocalizations during many of the same sampling points (n=21 sampling points, range in intervals between sampling: 2.4 – 93 hrs, median = 8.6 hrs). All of these behaviours were registered as binary events: present or absent during our observation.

We performed begging tests on day 3 of the experiment (average chick age: 3.82 days, s.e. = 0.10), 4 hours after their 11 am feeding. Each chick was placed alone into an empty cage and filmed for 120 seconds. During the time intervals of 30-60 seconds and 90-120 seconds, chicks were presented with forceps holding a small morsel of fish as was standard practice during hand-feeding. To trigger regurgitation of food, kittiwake chicks thrust upward toward the parent's bill and peck at it, much like in gull species (Cullem 1957) and in our experiment they performed a similar behaviour toward the forceps during feedings. Begging behaviour was later scored from videos by an observer blind to the chicks' treatment and included the number of pecks at the forceps and the number of thrusts toward the forceps and toward the general

direction of the observer. Begging vocalizations were difficult to distinguish reliably between chicks in the videos and were not analysed.

We also performed standardized sibling competition tests on the same day after all chicks were returned to their own cages together with their current sibling. The aim was to determine feeding order in a competitive setting. An observer blind to the chicks' identities presented chicks with 10-15 morsels of fish and scored which chick ate which morsel in the sequence they were delivered.

Statistical analyses

All statistical analyses were performed in the R statistical computing environment (R Development Core Team 2011). Differences in yolk androgens between first and second eggs of the same clutches were tested using paired t-tests. We tested effects of treatment on hatching success and chick mortality using 2 x 2 Fisher Exact tests. We performed an unpaired Wilcoxon rank sum tests to test effects of treatment on hatching time, hatchling size, and age of chick mortality.

All measurements of chicks were taken before they approached asymptotic body mass, so mass and bill length increased linearly with age, which we confirmed via visual inspection of the data. Growth data were, therefore, analysed using linear mixed effects regression (nlme package, Pinheiro et al 2011) with treatment as a fixed factor, age as a covariate and individual as a random factor.

Since aggression, submission and begging vocalizations were registered as binary variables, we analyzed these data using mixed effects binomial regression (lme4 package, Bates et al 2011). In all analyses testing effects of treatment on behaviour, we included only dyads that contained one chick from each treatment group and excluded the dyads containing two androgen chicks. In these models we included treatment as a fixed factor and chick age, residual body mass and mass differences between siblings as covariates. To compute residual body mass we regressed body mass on bill length for each chick, and used the model residuals as a proxy for the energy reserves of each chick. Mass differences between siblings were calculated for each chick as half of the mass difference between siblings: the larger chick was given a positive value, the smaller chick was given a negative value. We also tested for an effect of relative dominance on frequency of bouts of begging vocalizations, by computing half of the difference in dominance score (see below) between two siblings: the more dominant chick was given a positive value, the less dominant chick was given a negative value. In this model we included relative dominance and residual body mass as covariates. In all of the analyses of binary behavioural data, we included nest as a random factor because we were comparing chicks of the same dyad, and also chick because we used repeated measurements of the same chicks. Details about sample sizes are provided in the results section.

In certain dyads, chicks appeared to establish a dominance hierarchy more quickly and effectively than in other dyads. To determine whether elevated maternal androgen exposure produces more distinct dominance, we calculated a dominance score by dividing the frequency of observed aggression by the sum of the aggressive and submissive behavior shown by the chick. Then we compared these dominance scores between both siblings in a dyad and identified the sibling with the larger dominance score as the dominant chick in the dyad. We used a linear mixed model (including only dominant chicks) to test whether dominant androgen chicks had a higher dominance score relative to their sibling than did dominant control chicks, by using dominance score as the dependent variable and treatment as fixed factor. In these models we included only chicks coming from dyads that contained one androgen chick and one control chick. We excluded dyads in which neither chick showed aggression or submission because it was not possible to identify a dominant individual. In these linear mixed models, we included chick as a random factor to correct for repeated observations of the same dominant chicks in different dyads.

To test whether previous dominance status determines dominance status when paired with a new sibling, we performed a binomial mixed model in which we used previous

dominance status (1 = dominant, 0 = subdominant) as a predictor for dominance status in the subsequent dyad. The analysis included only dominance status from individuals in which we were able to calculate the dominance score for the current and subsequent dyad (i.e. in both dyads, we observed at least some aggression or submission). This data set included dyads including androgen and control chicks but also dyads including two androgen chicks. In these models, we included dyad and chick as random factors.

We analysed behavioural data from the begging tests using mixed effects Poisson regressions and in addition to the random factor of cage, we included an individual-level random effect that corrects overdispersion in the Poisson response variable (Maindonald and Braun 2003).

We performed linear mixed effects regressions to test whether androgen chicks took the first meals in a sibling competition test faster than control chicks did. Each competition test included a series of sequential trials (10 to15) which exceeds the number of fish morsels required to satiate a chick by ca. 40% (personal observation). We used trial number as the response variable in our models, and included cage as a random factor to correct for the fact that each competition test on the sibling pairs contained several trials. The predictor in the first model was the treatment of the winning chick and in the second model the relative dominance of the winning chick. The direction of the relationship indicates which chick fed to satiety first. For example a negative relationship would indicate that testosterone chicks (coded as 1) won more of the early trials (lower numbers) and fewer of the later trials (higher numbers) than did control chicks (coded as 0).

Results

Yolk androgens

Second-laid eggs contained substantially higher concentrations of testosterone ($t=5.451$, $df = 6$, $p = 0.0016$) and androstenedione ($t = 5.936$, $df = 6$, $p = 0.001$) than did first-laid eggs (Fig. 1a, 1b; see "Statistical analyses" in Materials and Methods for details about statistical tests).

Development, growth and survival

Hatching success did not differ between the two treatments (22 out of 40 androgen-injected eggs hatched, 17 out of 40 control-injected eggs hatched, $p = 0.371$). Androgen-injected eggs did not hatch significantly earlier than control-injected eggs ($W = 192$, $p = 0.898$). Hatchlings coming from androgen-injected eggs did not differ in mass ($W=237$, $p = 0.161$) or bill length ($W=179$, $p = 0.832$) from hatchlings coming from control-injected eggs.

We found no effect of treatment on mass (treatment: coef = 0.371, s.e. = 4.458, $df = 37$, $p=0.931$; age: coef = 4.223, s.e. = 0.184, $df = 251$, $p<0.001$) or on bill growth (treatment: coef = 0.768, s.e. = 0.625, $df = 37$, $p = 0.227$; age: coef = 0.999, s.e. = 0.016, $df = 251$, $p < 0.001$). Androgen chicks and control chicks showed similar mortality rates (Fisher Exact test: $p = 1.000$) and did not differ in the age at which they died ($W = 51$, $p = 0.160$, 11 controls vs. 14 androgen chicks).

Aggression

Chicks from androgen-injected eggs showed more frequent bouts of aggression than did chicks from control-injected eggs (coef = 2.128, s.e. = 0.516, $p < 0.001$, n=836 observations, 52 dyads, 39 individuals, Fig. 2a). Aggression decreased significantly with age (coef = -0.109, s.e. = 0.031, $p < 0.001$) but showed no relationship with residual body mass (coef = 1.030, s.e. = 1.232, $p = 0.403$) or mass difference between siblings (coef = 0.017, s.e. = 0.238, $p = 0.491$).

In an identical model in which we replaced mass asymmetry with bill length asymmetry we found qualitatively the same results: chicks from androgen-injected eggs showed more frequent aggression (coef = 2.063, s.e. = 0.513, $p < 0.001$), and aggression decreased with age

(coef = -0.108, s.e. =0.031, p < 0.001) but was not significantly predicted by residual mass (coef = 0.667, s.e. = 1.131, p = 0.555) or bill length difference between siblings (coef = 0.051, s.e. = 0.201, p = 0.807).

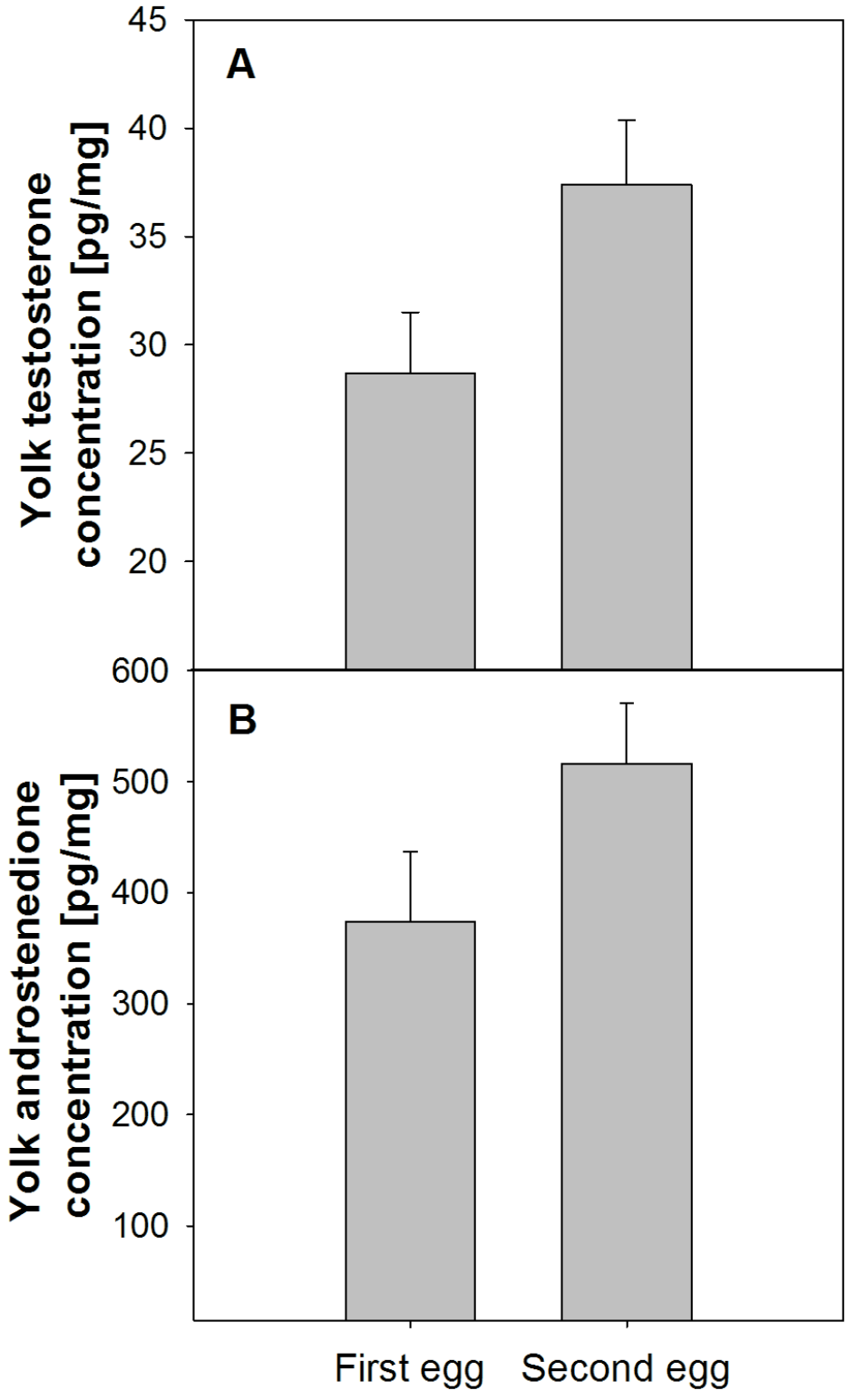

Figure 1: Means and standard errors of yolk testosterone (A) and androstenedione (B) concentrations in first and second kittiwake eggs.

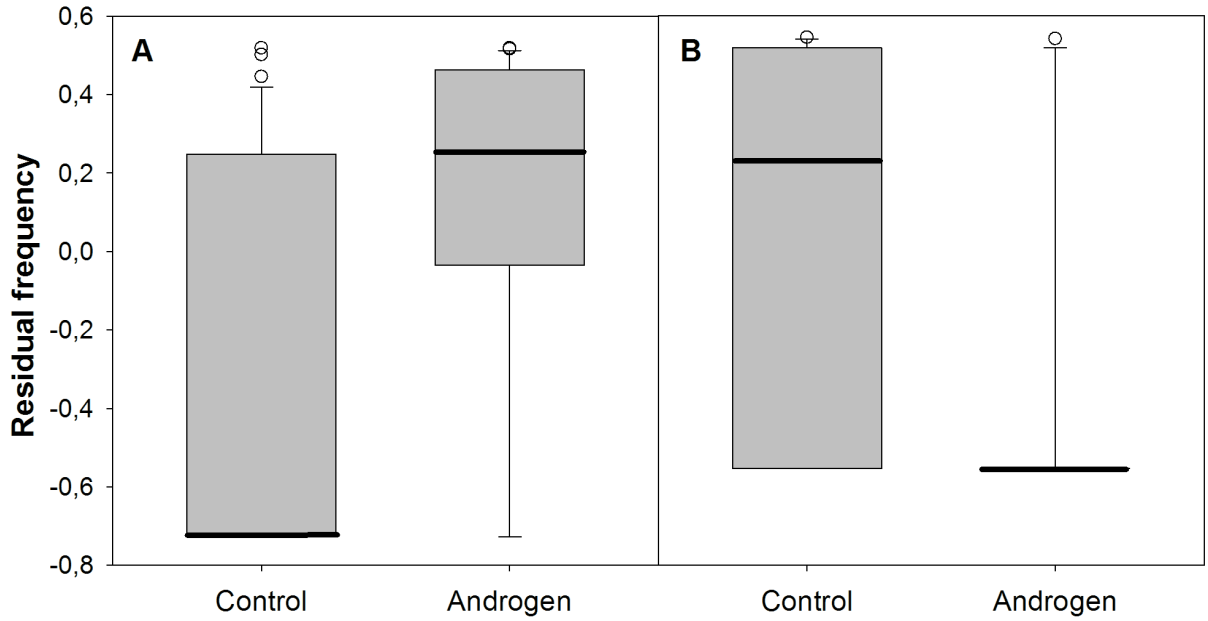

Figure 2: Residual frequency of bouts of aggression (A) and submission (B) in chicks coming from control and androgen-treated eggs. Residuals in figures produced by Poisson mixed effects regressions including individual and dyad as random factors.

Submission

We found a strong negative effect of androgen-injection on submissive behavior (coef = -2.176, s.e. = 0.579, $p < 0.001$, n=836 observations, 52 dyads, 39 individuals, Fig. 2b). In this model we found no significant association of submissive behavior with age (coef = -0.030, s.e. = 0.031, p = 0.351), residual body mass (coef = -2.013, s.e. = 1.448, p = 0.164), or mass asymmetry (coef = -0.021, s.e. = 0.028, p = 0.464).

In an identical model in which we replaced mass asymmetry with bill asymmetry we found qualitatively the same results: chicks from androgen-injected eggs showed less submissive behavior (coef = -2.110, s.e. = 0.5779, $p < 0.001$), but submissive behavior was not predicted by age (coef = -0.0317, s.e. = 0.0328, p = 0.334), residual body mass (coef = -1.589, s.e. = 1.323, p = 0.230), or bill length asymmetry (coef = -0.030, s.e. = 0.230, p = 0.895).

Dominance

We calculated a dominance score for all chicks and then calculated the difference in dominance score between siblings within dyads containing opposite treatments, and identified the chick with the higher score as the dominant chick in the pairing. Out of the 37 dyads in which chicks showed some aggression or submission, in 10 dyads (distributed over 7 individuals) the control chick was dominant, and in 26 dyads (distributed over 19 individuals) the androgen chick was dominant, and in one dyad, chicks were equally dominant. We found that dominant androgen chicks had a higher dominance score than did dominant control chicks (coef = 0.133, s.e. = 0.030, p = 0.0001, n = 36 dyads, 26 individuals). In a separate model we also found that dominant androgen chicks were relatively more dominant compared to their siblings than were control chicks (coef = 0.362, s. e. = 0.094, p = 0.0005, n = 36; Fig. 3). We also found that previous dominance status did not significantly predict current dominance status (coef = -0.0789, s.e. = 0.0701, p = 0.260, n = 36).

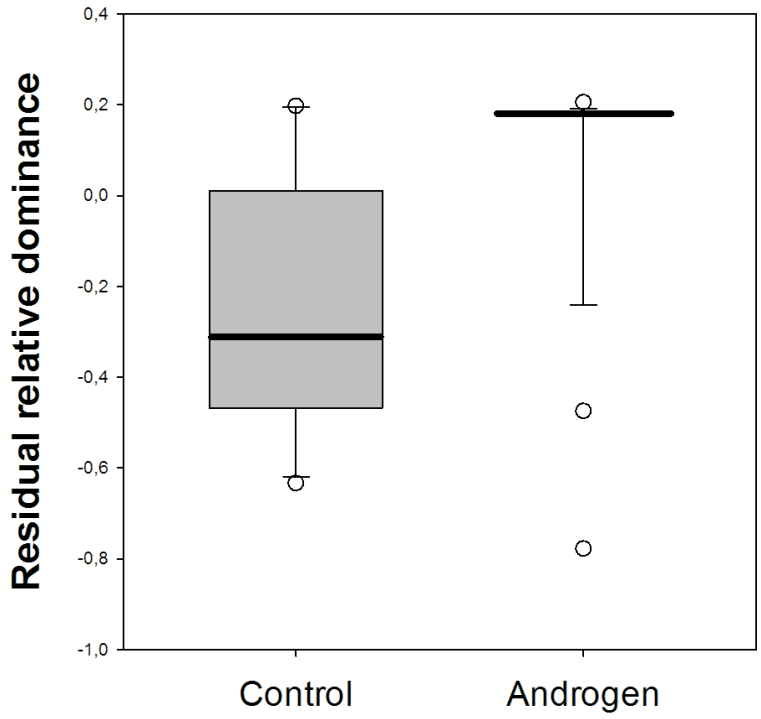

Figure 3: Residual relative dominance among dominant young in chicks coming from control and androgen-treated eggs. Residuals produced by linear mixed effects regressions containing individual as a random factor.

Begging

Androgen chicks did not perform more frequent bouts of begging vocalizations than did control chicks (coef = -0.159, s.e. = 0.455, p = 0.727, n = 386 observations, 31 pairings, 33 individuals, 102 total bouts of begging vocalizations). We also found no significant relationship between bouts of begging vocalizations and residual body mass (coef = -0.0133, s.e. = 0.017, p = 0.428) or mass differences among siblings (coef = 0.0192, s.e. = 0.0215, p = 0.372). In a second model, relative dominance showed no significant relationship with begging vocalisations (coef = 0.144, s.e. = 0.705, p = 0.829), nor residual body mass (coef = -0.018, s.e. = 0.018, p = 0.329).

In analyses of data from the standardized begging tests, frequency of begging pecks (coef = -0.183, s.e. = 0.527, p = 0.729, n =34) or begging thrusts (coef = -0.182, s.e. = 0.430, p = 0.6720) were not affected by treatment (mean pecks by control chicks = 26.15, s.e. = 7.24; mean pecks by T chicks = 22.62, s.e. = 4.46; mean thrusts by control chicks = 11, s.e. = 2.75; mean thrusts by T chicks = 12.52, s.e. = 2.91). In a second set of models, we also found no effect of relative dominance on begging pecks (coef = -0.210, s.e. = 0.631, p = 0.74) or begging thrusts (coef = -0.284, s.e. = 0.468, p = 0.543).

Competition for food

We found that within a competition test for food comprising several sequential trials, androgen chicks won more of the early trials and control chicks won more of the later trials (coef = -1.465, s.e. = 0.562, p = 0.01, n = 137 trials across 13 dyads, Fig. 4a). Then we used the same model but replaced the fixed factor of treatment with relative dominance. We found that higher relative dominance predicted early access to food even more strongly than treatment (coef = -0.058, s.e. = 0.012, p = 0.0001, Fig. 4b).

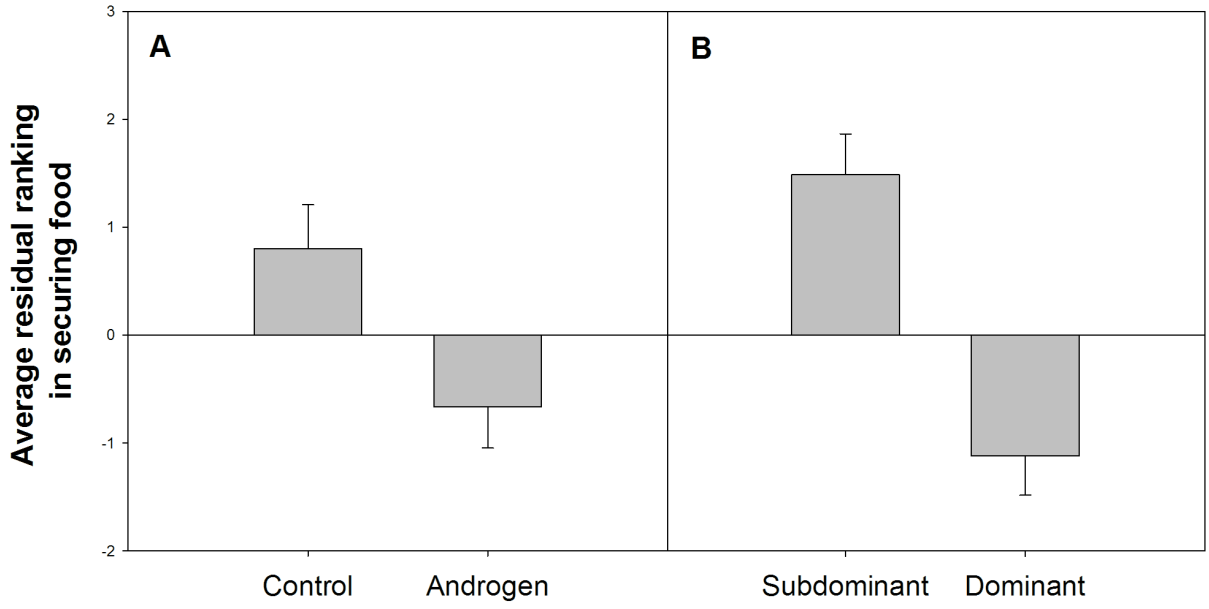

Figure 4: Residual ranking in competition for food in chicks coming from control and androgen-treated eggs (A) and in subdominant and dominant chicks (B). Ranking indicates the trial numbers (in a series of 10-15 trials) for which the focal chick succeeded in taking the food before its sibling. Therefore, a lower ranking value indicates more success in obtaining food in the earlier trials and a higher ranking indicates success in obtaining food only in the later trials. Residuals produced by linear mixed models containing cage as a random factor.

Discussion

Mothers are suspected to bestow phenotypic advantages on particular offspring to facilitate efficient reduction of the number of offspring in case of insufficient food (Mock and Parker 1997, 1998). Avian mothers systematically vary androgen concentrations over the laying sequence (Schwabl 1993, reviewed in von Engelhardt and Groothuis 2011), and the diversity in these patterns among species generated much interest in whether they correlate with particular reproductive strategies (e.g. hatching asynchrony adjustment hypothesis: Groothuis et al 2005). For example, it has been suggested that a pattern of decreasing yolk androgens over the laying sequence of eggs within a clutch might support siblicide-mediated brood reduction as elevated yolk androgens may help the oldest dominant chick outcompete the younger ones (Schwabl et al 1997). We tested this for the first time by combining a comparative and experimental approach, using the facultatively siblicidal black-legged kittiwake. Our results do not support this hypothesis.

First, we found that yolk androgens did not decrease, but increased steeply, over the laying sequence (Fig. 1) as was reported in Vallarino et al. (2012). This pattern fits the idea that mothers compensate delayed hatching in marginal young by boosting their competitive ability (e.g., Groothuis et al. 2005; Muller and Groothuis, in review). Clearly, this does not fit the idea that siblicidal species like the kittiwake use yolk androgens to aid core young in eliminating marginal young, as has been implied for the cattle egret (Schwabl et al. 1997). In kittiwakes, first-hatching core chicks inevitably assume the dominant position in the social hierarchy usually within 24 hours of the marginal chick's hatching (Braun and Hunt 1983) so hatching position, and not yolk androgens, seem to determine dominance position within the brood. In fact, in all siblicidal species, dominance hierarchies always correlate perfectly with hatching sequence (Drummond 2006), whereas dominance hierarchies do not consistently correlate with

a species' pattern of yolk androgens over the laying sequence (decreasing pattern: cattle egret, Schwabl et al. 1997, blue-footed booby, Dentressangle et al. 2008; flat pattern: brown booby and blue-footed booby, Drummond et al. 2008; increasing pattern: blue-footed booby, Dentressangle et al. 2008, black-legged kittiwake, Vallarino et al. 2012, Australian pelican, G. Johnston, unpublished data). Clearly, the size and developmental advantage of core young supersedes any potential effects of yolk androgens in determining dominance during the early nestling period.

Given the observed pattern of increasing yolk androgens in the kittiwakes, one would expect that (1) similar to other species with increasing patterns, kittiwake mothers might allocate more maternal androgens to marginal young to increase their competitive ability to access resources, which may manifest itself in enhanced growth, begging, or scrambling for food; (2) elevated yolk androgens do not increase aggressive behaviour, otherwise it would counteract the establishment of a clear dominance rank order in the nest, undermine efficient siblicide in the event that siblicide occurs, and intensify aggressive interactions among the siblings. While we found some evidence for (1), our results clearly stand in contrast to (2).

With regard to (1), experimentally elevated androgens affected chick performance positively in scramble competition for food, but we found no effect of this treatment on growth. This may be due to the fact that apart from the one sibling competition test, chicks were hand-fed individually and siblings did not have to compete for food. However, we also found no effect of treatment on the frequency with which androgen and control chicks performed bouts of begging vocalizations in their home cages. From a functional point of view, the latter finding might not be surprising as vocal begging cues have been suggested to be most important for determining the overall level of brood provisioning (although they sometimes also affect food allocation within broods, e.g. Kilner et al 1999). Forbes (2007) suggested that if intensity of a brood's vocal begging determines the amount of food delivery, then core siblings might actually benefit from vocal begging by marginal chicks (Forbes 2007, Kilner et al 2004). Furthermore, if begging vocalizations signal the hunger level of a chick honestly to parents, maternal androgens should not interfere with such essential offspring communication to parents. Indeed, several experimental studies have failed to find an effect of yolk androgen exposure on begging vocalizations (Eising et al 2003, Pilz et al 2004).

There was also a lack of a treatment effect on begging in the begging tests. This might be due to the fact that we tested the chicks alone, outside the context of sibling competition. Previous experimental studies demonstrated that yolk androgen exposure enhances bill gaping and vigor of begging shown by posture, as well as competitive ability (Eising et al. 2003, von Engelhardt et al. 2006, Pilz et al. 2004, Schwabl et al. 1996). In general, such conspicuous visual displays are considered more important in determining within-brood allocation of food (Muller and Smith 1978, Bengtsson and Ryden 1983, Leonard and Horn 2001, Leonard et al. 2003). But these positive effects of yolk androgen exposure on actions involved in scramble competition manifest themselves mostly when chicks are tested in the presence of their siblings (e.g., canaries: Schwabl 1996; European starlings: Pilz et al. 2004; black-headed gulls: Eising et al. 2003) but not when chicks are tested alone (canaries: Müller et al. 2010, European starlings: Pilz et al. 2004; yellow-legged gulls: Boncoraglio et al 2006; but see von Engelhardt et al. 2006). Indeed, when we measured begging effort via standardized solitary begging tests, we found no effect of androgen treatment on begging pecks or begging thrusts. But when chicks were presented with food in their home cages in the presence of their sibling, androgen chicks performed better than control chicks in securing the first meals. This finding supported the first expectation, and suggested that kittiwake mothers might deposit higher yolk androgens into second eggs to help marginal chicks compete for food.

Despite the increase of androgens over the laying sequence, experimentally elevated yolk androgens strongly increased aggression and dominance status, clearly in contrast with the second expectation. Also, control chicks showed much more frequent submissive behaviour in response to aggression than did androgen chicks. Core young in siblicidal species use aggression towards marginal siblings to secure dominant status (Drummond 2006) which gives them power to control food distribution within the brood. Indeed, social dominance in the sibling dyads turned out to be an even better predictor of who secured the first meals than androgen

treatment. These findings create a problematic paradox: in a natural setting, early-hatching core chicks, which are exposed to lower yolk androgens, inevitably assume the dominant role due to their larger size and have the power to control food distribution and kill their subdominant siblings (Drummond 2002, 2006), yet in our experiment eliminating hatching asynchrony, we found that the higher yolk androgen exposure characteristic of marginal chicks largely determines dominance. Why then do mothers allocate higher yolk androgens to marginal young if they can never reap the benefits for sibling competition due to their inferior social rank determined by delayed hatching?

We suggest an avenue by which benefits of higher yolk androgen exposure may manifest after marginal young become victims of siblicide. In poor years, marginal young are almost always ejected from the nest following an onslaught of violent attacks by the core sibling (Cullen 1957). Kittiwakes live in large dense colonies situated on cliffs in which rows of nests sit along stratified horizontal ledges of rock (Cullen 1957). Siblicidal attacks from the core offspring force marginal offspring out of the nest and into adjacent nests or into nests below (Roberts and Hatch 1994). Kittiwakes have high rates of adoption, at least 8% or higher (Pierotti and Murphy 1987, Roberts and Hatch 1994; Helfenstein et al 2004), facilitated by kittiwake parents accepting foreign chicks of various ages in their nests (Cullen 1957, Roberts and Hatch 1994). Vagrant chicks readily enter many foreign nests (Roberts and Hatch 1994) and respond indiscriminately to any adult (Storey et al 1992). Ejected marginal chicks have been observed to enter nests in which the resident chicks are of similar or smaller size, expel resident chicks, and then survive in the foster nests until fledging (Pierotti and Murphy 1987, Roberts and Hatch 1994). This fits very well with our finding that subdominant social status in the natal nest does not undermine a chick's prospects of achieving dominance in a new nest containing a different chick. Higher maternal androgen exposure should increase their odds at winning such contests for dominance. An 8% adoption rate indicates that, even after siblicide, ejected marginal chicks retain substantial reproductive value and mothers stand to gain a large increment in fitness via adoption of their marginal chicks without incurring associated rearing costs.

Müller and Groothuis (in press) suggested that mothers aiming for survival of the whole brood should produce an increasing pattern of yolk androgens which would promote the survival of marginal chicks in good years. We also show that flat or decreasing patterns of yolk androgens occur in species that produce marginal chicks primarily for insurance, in which marginal chicks merely act as replacement units for failed core eggs or chicks. According to this model, black-legged kittiwakes, which show a strong increase in yolk androgens over the laying sequence, should reap more reproductive value from survival of marginal young alongside the core ("extra" reproductive value, Mock and Parker 1997) compared to insurance value. Data from Braun and Hunt (1983) indicate that marginal kittiwakes do contribute more extra reproductive value (0.37), than insurance value (0.045, see Mock and Parker 1997 for methods of calculation). In addition, we suggest that adoption represents a third component of their reproductive value that has thus far been overlooked. Adoption occurs at high rates in many other colonial seabirds as well (e.g., herring gull: 5-35% of pairs, Graves and Whiten 1980; ring-billed gull: 4-38% of pairs, Brown et al. 1995; common gull: 23.4% of pairs, Bukacinski et al. 2000; Audouin's gull: 18.5 -48%, Oro and Genovart 1999; common tern: 15-24% of pairs, Morris 1988; Little tern: 29% of pairs, Saino et al 1994) but also in other birds (e.g. American avocet: 19.8-32.2% of pairs, Lengyel et al. 1998; Greylag goose: up to 50% of pairs, Kalmbach et al. 2005) and at various stages of development (nest-switching at fledging, e.g. 5.4 % of Eagle owlets, Penteriani et al 2008, Eastern bluebird: Ligon et al 2009, Plissner et al 1999). Many of these avian families show increasing patterns of yolk androgens over the laying sequence (e.g. gulls: Eising et al. 2001, Verboven 2003, Rubolini et al. 2011, tern: French et al. 2001; owl: Hahn 2011, Eastern bluebird: Navara 2006), suggesting that higher yolk androgen exposure in marginal young may enhance the odds that mothers might profit from having a marginal offspring successfully reared in a foreign nest. We therefore propose the "adoption facilitation hypothesis" which states that mothers deposit relatively more yolk androgens into marginal eggs to increase the survival prospects of last-hatching young by enhancing their ability to appropriate parental care from unrelated adults.

Acknowledgments

We gratefully thank Thierry Boulinier for helping with egg collection, Roald Hansen for extensive logistical support in Ekkeroy, Bonnie de Vries and Ilse Weites for assistance with hormone assays, Gerard Overkamp for help in setting up experiment rooms, and D. J Anderson for helpful comments on the manuscript.

Chapter 5. Testosterone increases siblicidal aggression and begging in black-legged kittiwake chicks (*Rissa tridactyla*)

Unpublished manuscript

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Abstract

To compete for parental food deliveries nestling birds have evolved diverse behaviours such as begging displays and sibling aggression. Testosterone (T) has been implicated as an important mechanism orchestrating such competitive behaviours, but evidence is scarce and often indirect. Siblicidal species provide an interesting case in which a clear dominance hierarchy is established and the dominant chicks may lethally attack siblings. We experimentally elevated T in chicks of a facultatively siblicidal species, the black-legged kittiwake, *Rissa tridactyla*, and showed that T-treated chicks were more aggressive toward their sibling than were control chicks. In such facultatively siblicidal species, chicks normally exhibit intense aggression only when threatened by starvation. Indeed, we found that chicks in relatively poorer condition were more aggressive than were chicks in better condition, even among T-treated chicks, suggesting the action of an additional signal modulating aggression. Relatively larger siblings were also more aggressive than were relatively smaller siblings, confirming the importance of size and developmental advantage in determining dominance hierarchies within the brood. In addition, T increased aggression toward a simulated predator, indicating that T can increase aggression in contexts other than siblicide. Although T also promoted aggression-mediated dominance, we were able to separately examine the effects of T treatment and dominance and show for the first time that both factors independently increased begging.

Introduction

Avian parents can afford to bring only limited provisions to the nest because foraging is costly, so nestlings face the challenge of acquiring enough food for subsistence and essential processes such as growth (Dunn 1980) and immune function (Saino & Møller 2002; Lee 2006). This struggle for nourishment propelled the evolution of complex behaviours in nestlings centred on securing food, such as begging displays toward parents (Kilner 2002), as well as competition among nest mates (Glassey & Forbes 2002). But the degree to which nestlings express these behaviours depends strongly on their social and environmental milieu. The neuroendocrine system integrates such social and environmental cues, facilitates the appropriate behavioural response, and then releases hormones into the bloodstream to coordinate the relevant tissues involved in executing the response (Lessells 2008).

Testosterone (T) was relatively recently implicated as one of the primary hormones that facilitate begging and competitive behaviours in nestlings (reviewed in Ros 2008; Smiseth et al. 2011). In many avian taxa chicks limit their behaviour to begging displays and scrambling for food, but in a diverse set of species, siblings fiercely attack each other, establishing a linear dominance hierarchy in the nest in which dominant young often kill one or more subdominant young (Drummond 2006). Several studies have provided indirect evidence that T might also be an important proximate cause in facilitating sibling aggression. In the siblicidal white stork, *Ciconia ciconia*, circulating T is higher in behaviourally dominant first-hatching chicks (Sasvari et al. 1999) compared to later-hatched subdominant chicks, and it is especially high in dominant chicks coming from nests with siblicide (Sasvari et al. 1999). In siblicidal boobies, genus *Sula*, baseline nestling T is low (Nuñez de la Mora et al. 1996; Ramos-Fernandez et al. 2000; Tarlow et al. 2001), but temporarily increases to a high level during siblicidal aggression (Ferree et al. 2004). Also, obligately siblicidal Nazca booby, *Sula granti*, chicks have higher T than do faculatively siblicidal blue-footed booby, *S. nebouxii*, chicks (Müller et al. 2008). But until now, a causal role for T in siblicide was never directly tested. In our study, we experimentally increased circulating T concentrations in facultatively siblicidal black-legged kittiwake (*Rissa tridactyla*, kittiwake hereafter), nestlings to test whether it increases sibling aggression.

In siblicidal birds, dominant chicks receive a larger portion of the food by intercepting food transfers intended for subdominant chicks and by controlling the timing, the location in the nest, and the intensity with which subdominant chicks can beg (Drummond 2002). The superior efficacy of begging in dominant chicks might simply be due to the dominants' aggression inhibiting begging behaviour in subdominant chicks. Alternatively, dominant chicks might beg more vigorously than subdominant chicks because the T up-regulation associated with aggression also increases begging intensity. Manipulation of T to study begging performance has only been carried out in two species, both non-siblicidal. In the pied flycatcher, *Ficedula hypoleuca*, T increases the intensity of competitive begging (Goodship & Buchanen 2007). But in the black-headed gull, *Larus ridibundus*, T decreases begging and increases aggression (Groothuis & Ros 2005). However, in the latter species begging displays are a cooperative action of the whole brood and behaviourally distinct from the individual competition for food regurgitated on the ground by parents. This opens the possibility that T only facilitates begging in a competitive context. As the kittiwake is a relatively closely related species to the black-headed gull, but with begging performed in a competitive context, it is an interesting study species for testing the effect of T on this behaviour. Also, in contrast to the kittiwake, in black-headed gulls aggression is never directed at brood mates like in siblicidal species but only toward chicks and adults from other nests. In this study, we tested the effect of T on aggression and dominance status in kittiwake chicks in the field and disentangled their separate effects on begging behaviour. As dominance hierarchy is influenced by size (Drummond 2006), and sibling aggression (Drummond & Chavelas 1989) and begging (Price et al. 1996) are influenced by body condition, we take both size and body condition into account in our analyses.

Methods

Study area, species and experimental design

We studied kittiwake nestlings in a colony on the Ekkerøy peninsula in Norway (70°N, 30°E), containing ca. 20,000 breeding pairs. The colony is situated on a south-oriented vertical cliff rising 40-50 meters from the Varanger fjord and stretches ca. 1 km along the coast. Kittiwakes lay a modal clutch size of 2 eggs (Jacobsen et al. 1992, Hatch et al. 2009) with an average laying interval of 2.5 days between eggs (Hatch et al. 2009). In this species, the second chick hatches ca. 1.3 days after the first chick does (Hatch et al. 2009, Braun & Hunt 1983). Young fledge ca. 42-44 days after hatching (Hatch et al. 2009).

We selected nests with only one chick to avoid that our T manipulation might induce siblicidal behaviour in natal nests. We observed competitive behaviour in foster nests where two chicks were placed together for 90 minutes. In addition, two standardized behaviour tests were conducted in the natal nest (see below). We were able to select 96 nests (1.5 - 4.5 m from the ground, reachable by ladder) containing one chick. From these nests we selected 32 experimental chicks estimated to be between 7-14 days old, the age of peak siblicidal behaviour (White et al 2010) and younger than the age at which chicks develop individual recognition that would otherwise hamper our cross-fostering (see below). Age was estimated based on body mass (mean mass: 203g, s.e = 8.9; see Barrett & Runde 1980, Gabrielsen et al 1992, Barrett 1996 for growth curves).

Testosterone manipulation

We created silastic capsules made out of silicon tubing (12 mm long, 1.0 mm inner diameter, 3.0 mm outer diameter). In 16 chicks, the capsules were packed with crystalline T (Sigma, Germany) and in the remaining 16 chicks, implants were empty. We sealed the implants with silicon glue (1mm of the tube filled with glue on each end, so 10 mm filled with T) and placed them into saline solution for at least 24 hours. The plasma samples for validating T treatment for this study were destroyed in transport, but our lab has used an identical implantation dose and protocol in chicks of the closely-related black-headed gull which have similar concentrations of baseline T (kittiwakes: < 0.01 - 0.35 ngT/ml plasma, Kitaysky et al. 1999; black-headed gulls: <0.01 - ca. 0.4 ngT/ml, Ros et al. 2002). In our black-headed gull studies, the implantation protocol yielded very consistent T elevation within the physiological range (mean and SE of controls: 0.10 ± 0.06 ng T/ml, n =9, chicks with T implants: 0.26 ± 0.03 ng T/ml, n = 15, Ros et al. 1997; controls: 0.08 ± 0.01 ng T/ml, n =5, chicks with T implants: 0.24 ± 0.03 ng T/ml, n = 8; chicks after T implants were removed: 0.07 ±0.04, n = 8, Ros et al. 2002) and plasma T elevation did not differ with age at which chicks were implanted (A. Ros, unpublished thesis).

We implanted the capsules subcutaneously in the neck region after disinfecting the skin and applying the local anaesthetic lidocaine (Xylocaine, NL). The incision was closed with dissolvable stitches and wound glue. All implants were removed from the chicks at the end of the experiment.

Ethical note

The experimental protocol was approved by the The Norwegian Animal Research Authority under licence number 2644. All implanted chicks were monitored daily and weighed at implantation, on the day of behavioural observations and on the day implants were removed. Implantation caused no loss of body mass, infections or mortality. During implantation, each chick was wrapped in a towel to inhibit movement, and the head was covered with another towel which visibly reduced tension and resistance in the animal. We took 150 µl of blood from each chick, which falls well within the guidelines for maximum volume for a single sample from an animal with a body mass of 100 g or more.

Presence of investigators on the ground do not disturb this cliff-dwelling species as most nests were between 3 and 50 m above the ground. Adults only flew away when we climbed up to their nests with a ladder. During such disturbances, adults only flew in a small circle above the colony and almost immediately returned to the nest. We never observed any loss of eggs or chicks due to parents leaving the nest for these short flights. Nevertheless, to minimize disturbance we monitored the nests using a 4 m pole with a mirror attached to it, which allowed us to get a close look at chicks without causing parents to fly away from the nest. We only climbed up to nests when we needed to collect chicks for measurements, surgery or behavioural observations.

Aggressive behaviour in kittiwakes can range from mild aggressive displays in which dominant chicks lunge towards their sibling and often don't even make contact, to severe aggression that damages feathers. Although chicks lack the weaponry and strength to inflict serious injuries on their siblings, prolonged exposure to severe aggression would cause substantial distress to the victim. Aggression among chicks usually begins with mild aggression, eventually escalates to severe aggression and culminates in ejection. To limit the progression from mild aggression to severe aggression we paired chicks in the observation nest for only 90 minutes. We intended to intervene if aggression became severe by terminating the observations and observing that pair again on the following day, but this was never necessary. The maximum aggression we observed was relatively frequent pecks directed toward the back or lower nape of the neck of a sibling near the end of the observation period but it never escalated to the point in which aggression caused feather damage. Although during our observations none of the chicks were ejected from the nests, we chose observation nests that were located just above a ledge, so any ejected chicks would have fallen no more than 15 cm.

In the standardized behavioural tests, we used a stuffed dummy chick that we made ourselves using a freshly dead (ejected) chick found on the ground in the colony.

Sibling pairing and behaviour observations

Behaviour observations began ca. one week after chicks were implanted (mean: 7.1 days after implantation, SE = 0.24), to allow sufficient time for T to enter the circulation and for incisions to heal. For each behaviour observation, we paired one T chick and one control chick with size differences within the natural range for siblings. Kittiwake broods begin with a 17.5-24 % mass advantage in senior chicks (Braun & Hunt 1983, M. Müller, unpublished data). Based on this initial mass advantage and differential rates of growth among senior and junior chicks (Braun & Hunt 1983), the proportionate size difference between senior and junior chicks remains fairly consistent over time (estimated from Braun & Hunt 1983: 21-27% mass difference throughout the nestling period depending on the breeding conditions). In our experiment, we paired T and control chicks taking care that size differences between siblings did not exceed the natural range (mean percentage mass difference: 16.4%, SE = 2.3).

T chicks used in the experiment had an average body mass of 230.9 g (95% CI: 209.3 to 252.6) which did not differ significantly from the average body mass of control chicks (mean: 250.8 g, 95% CI: 223.4 to 278.2). At implantation, chicks were randomly assigned to one of the two treatment groups and were then paired so that in 9 of the sibling dyads, the control chick was slightly larger, and in 7 of the dyads the T chick was slightly larger (average size advantage of controls over T chicks across all dyads: 7.4 %, 95% CI: -3.3 to 18.0%, therefore not significantly different from zero).

We performed behaviour observations by transferring chicks to two easily observable nests in which parents were rearing chicks of a similar age. For the duration of the observation period, we placed one experimental chick dyad into each of the two nests and placed resident chicks into foster nests. Kittiwake parents readily accept and feed foreign chicks of various ages in their nests (Cullen 1957, Roberts & Hatch 1994) and chicks respond indiscriminately to any adult (Storey et al. 1992) during the first three weeks of age (personal observation). Chicks also do not react differently toward foster siblings than toward their own siblings (personal observation).

We observed each chick dyad for 90 minutes and counted the total number of aggressive pecks directed at a sibling and the total number of begging pecks directed at the parent during the observation period. During observations of all but three dyads, parents attended the nest during part of the observation period (in those nests, mean parental attendance: 78% of the time, s.e = 12.6 %). At the end of each behaviour observation, chicks were weighed and measured.

Standardized behaviour tests

On the day after chicks were observed with a sibling and just before removing the implants, we performed two standardized behaviour tests on chicks in their natal nest. First, we tested sibling aggression by presenting chicks with a dummy kittiwake sibling just outside of the nest for 30 seconds, then placed it in the nest for 30 seconds, then used it to peck the resident chick three times, and then left it in the nest for another 30 seconds. We recorded the total number of times the resident chick pecked the dummy chick. This dummy consisted of a stuffed chick of about 12 days old, fixed on a stick of 1m length. Then, to test anti-predator aggression, we performed a similar test but now using the observer's hand, and recorded the number of pecks toward the observer's hand. At this point chicks had been handled several times by investigators and moved away or attacked when approached by human hands. We performed the sibling aggression test before the predator aggression test in half of the chicks, and vice versa in the other chicks.

Blood samples

All experimental chicks were blood-sampled at the end of the behaviour observation period via brachial veni-puncture using 25 gauge needles and heparinized capillary tubes for collecting the blood which was then put into Eppendorf tubes. Blood was centrifuged for 10 minutes at 3300 rpm and then plasma was pipetted into a separate tube. Red blood cells were stored in 70% EtOH and refrigerated until DNA extraction. Red blood cells were used to molecularly sex chicks using Chelex for DNA extraction and PCR amplification of the sexually dimorphic CHD locus (Griffiths et al. 1998; Goerlich et al. 2009).

Statistical Analyses

We performed all statistical analyses in the R statistical computing environment (R Development Core Team 2011). All tests were two-tailed with significance delimited by alpha = 0.05. Each model represented a simultaneous test of the statistical significance of all predictor variables together and was not followed by stepwise elimination of non-significant predictors to avoid inflating Type I error due to multiple testing (Mundry & Nunn 2009).

To test whether T treatment influenced the number of aggressive pecks directed toward a sibling, we performed a Poisson mixed model including treatment and sex as fixed factors, size differences among siblings (see below), residual body mass as a proxy for body condition (see below), and body size (see below) as covariates, and sibling dyad as a random factor (lme4 package, Bates et al. 2011). Then we performed an additional mixed model including all of the same predictors but also two interactions: 1) an interaction between treatment and residual body mass, to determine whether residual body mass influenced aggression only in control birds or also in T-treated birds and 2) an interaction between treatment and size differences between siblings, to determine whether T increased aggression only in relatively larger siblings or also in relatively smaller siblings. In all Poisson mixed models we also included an individual-level random effect that corrects over-dispersion in the Poisson response variable (Maindonald & Braun 2003).

To quantify the size of chicks, we performed a principle components analysis on our three body measurements of mass, tarsus length and bill length which produced a linear combination (Bookstein 1989) of the three size variables (first principal component: 0.863

proportion of the variance). Size differences between siblings were calculated for each chick as half of the size difference between siblings: the larger chick was given a positive value, the smaller chick was given a negative value. To compute residual body mass we regressed body mass on bill length for each chick, and used the model residuals as a proxy for the energy reserves of each chick.

To test whether T increases begging directly or via its effects on social dominance, we performed the same Poisson mixed model on the number of begging pecks as we used to analyze the number of aggressive pecks but used a restricted dataset that included only sibling dyads in which chicks clearly established a social dominance hierarchy (n = 13 dyads instead of 16). In these models we included dominance rank as a fixed factor in addition to the other predictors. We defined dominance rank as a binary variable by identifying the dominant sibling as the sibling that showed the most aggression. In two dyads, neither chick showed any aggression so we could not determine dominance. In one other dyad, chicks did not differ substantially in aggression (3 aggressive pecks vs. 2 aggressive pecks) so we could not confidently determine dominance. In two other nests both chicks showed some aggression but in both cases the dominant chick was easy to identify as it showed more than 8 times more aggression than its sibling. In all remaining nests the dominant chick was easy to identify because only one chick showed any aggression at all. We performed the same model (minus the predictor of dominance rank) on all 16 dyads and results did not qualitatively differ, so we only reported the results for the analyses of the 13 dyads described above.

We also analysed the number of aggressive pecks chicks performed during the standardized behaviour tests in which they were presented with a dummy chick or a simulated predator. First, to test whether treatment and body condition influenced chick aggression differently depending on the stimulus, we performed a Poisson mixed model on the number of aggressive pecks performed by chicks in reaction to both stimuli. This model included the two interaction terms treatment*type of stimulus (sibling or predator) and residual body mass*type of stimulus; the main effects of treatment, residual body mass, type of stimulus and sex; and chick and observation number as a random factor. Then we performed another Poisson mixed model on the same data set including the same predictors but excluding the two interactions so that we could test whether chicks reacted more aggressively toward one stimulus, and whether there was an overall effect of treatment and residual body mass on aggression across all tests. Then we split the dataset and analysed data from the two behaviour tests separately again using Poisson mixed models. In these analyses we included treatment and sex as fixed factors, residual body mass a covariate and chick as a random factor.

Results

Aggression within sibling pairs

T-treated chicks performed more aggressive pecks toward their siblings than control chicks did ($b = 2.4$, $SE = 1.13$, $p = 0.03$, Fig. 1A). Chicks with low residual body mass performed more aggressive pecks than did chicks with high residual body mass ($b = -0.07$, $SE = 0.02$, $p = 0.002$, Fig. 1B). The number of aggressive pecks also correlated positively with relative size advantage in the nest ($b = 5.58$, $SE = 1.58$, $p < 0.001$, Fig. 1C), but showed no relationship with sex ($b = 0.78$, $SE = 1.23$, $p = 0.53$) or overall body size ($b = -0.03$, $SE = 0.35$, $p = 0.94$).

We then performed an identical model that included also an interaction between T treatment and residual body mass, and an interaction between T treatment and size difference between siblings. The interaction between T treatment and residual body mass was not significant ($b = -0.095$, $SE = 0.081$, $p = 0.24$; main effect residual body mass: $b = -0.028$, $SE = 0.039$, $p = 0.47$, main effect treatment: $b = 4.58$, $SE = 2.81$, $p = 0.10$) suggesting that poor body condition increased aggression in the same way for control chicks and T-treated chicks. The interaction between T treatment and size difference between siblings was also not significant ($b = 11.94$, $SE = 7.40$, $p = 0.11$; main effect size difference between siblings: $b = 1.8$, $SE = 2.25$, $p = 0.42$) indicating that T treatment increased aggression in siblings that were not only relatively

larger in size but also siblings that were relatively smaller in size. In this model overall size (b = -1.26, SE = 0.82, p = 0.12) and sex (b = 8.80, SE = 5.05, p =0.08) were again not significant predictors.

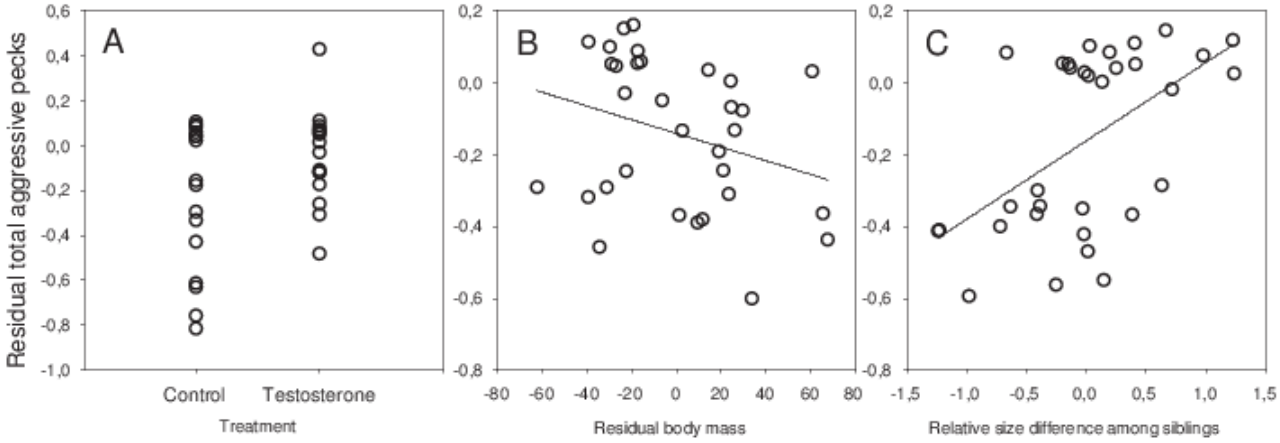

Figure 1: Residual total aggressive pecks performed during the observation period, in relation to (A) testosterone treatment, (B) residual body mass, and (C) relative size differences between sibling pairs. In each figure, the residuals were produced from a Poisson mixed model containing the predictors: testosterone treatment, residual body mass, size differences between sibling pairs, size of the focal chick, and sex, minus the predictor represented on the x-axis. All three models contained also sibling dyad and observation number as a random factor.

Standardized aggression tests

We found that T treatment increased the number of aggressive pecks towards stimuli presented during the behavioural tests, and chicks performed more aggressive pecks toward a dummy chick than toward a simulated predator (Table 1A). The number of aggressive pecks showed no significant relationship with residual body mass (Table 1A).

The effect of T treatment on the number of aggressive pecks performed by chicks did not depend on whether the stimulus was a dummy sibling or a simulated predator (Table 1B). The effect of residual body mass on the number of aggressive pecks performed by chicks, however, depended on stimulus type (Table 1B). Sex was not a significant predictor in this model (Table 1B).

In an analysis of behavioural response to a dummy intruder chick, T treatment increased the number of aggressive pecks (Fig. 2A), and like during the sibling dyad observations, chicks with lower residual body mass performed more pecks than did chicks with higher residual body mass (Fig. 2B), whereas sex was not a significant predictor (Table 1C). In the analysis of the response to the predator stimulus, T treatment again increased the number of pecks, but aggressive pecks showed no relationship with residual body mass (Table 1D).

Begging

Both dominant (b = 7.43, SE = 3.08, p = 0.02, Fig. 3A) and T-treated chicks (b = 7.23, SE = 3.08, p = 0.02, Fig. 3B) performed more begging pecks than did subdominant or control chicks. In this model, males begged significantly more than females did (b = 6.73, SE=3.31, p = 0.04) and begging correlated negatively with residual body mass (b = -0.10, SE = 0.05, p = 0.0497). Size differences among siblings (b = 4.61, SE = 3.24, p = 0.16) and overall size (b = -0.29, SE = 0.64, p = 0.66) were not significant predictors of begging in this model.

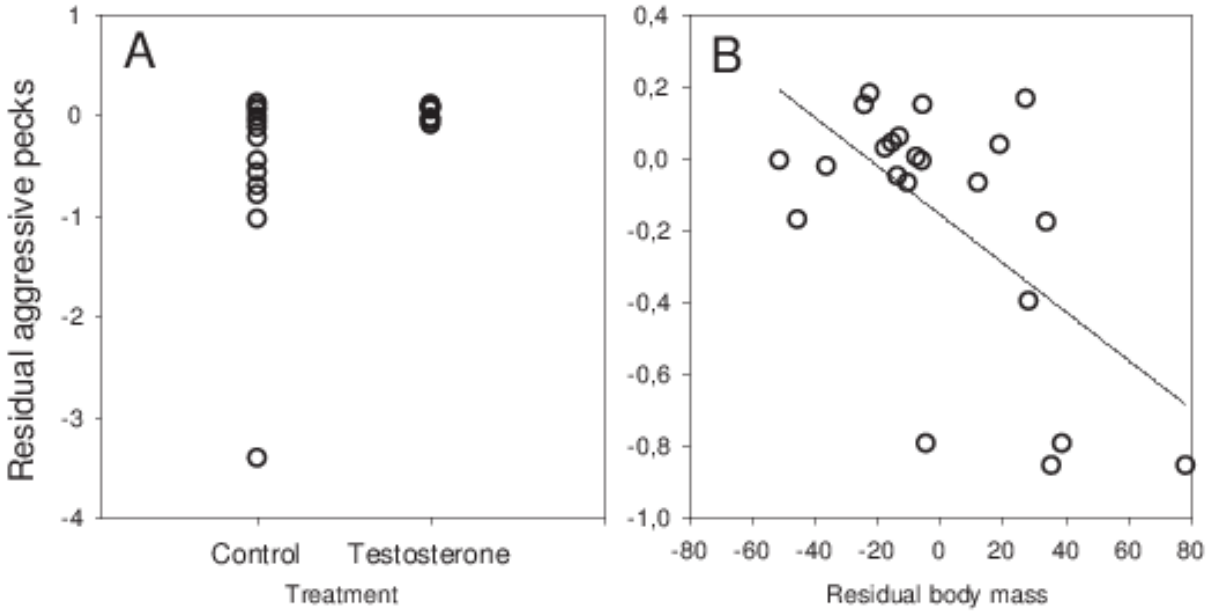

Figure 2: Residual total aggressive pecks directed toward a dummy chick in relation to (A) treatment, and (B) residual body mass. In both figures, the residuals were produced from a Poisson mixed model containing the predictors: testosterone treatment, residual body mass, and sex, minus the predictor represented on the x-axis. Each model contained also observation number as a random factor.

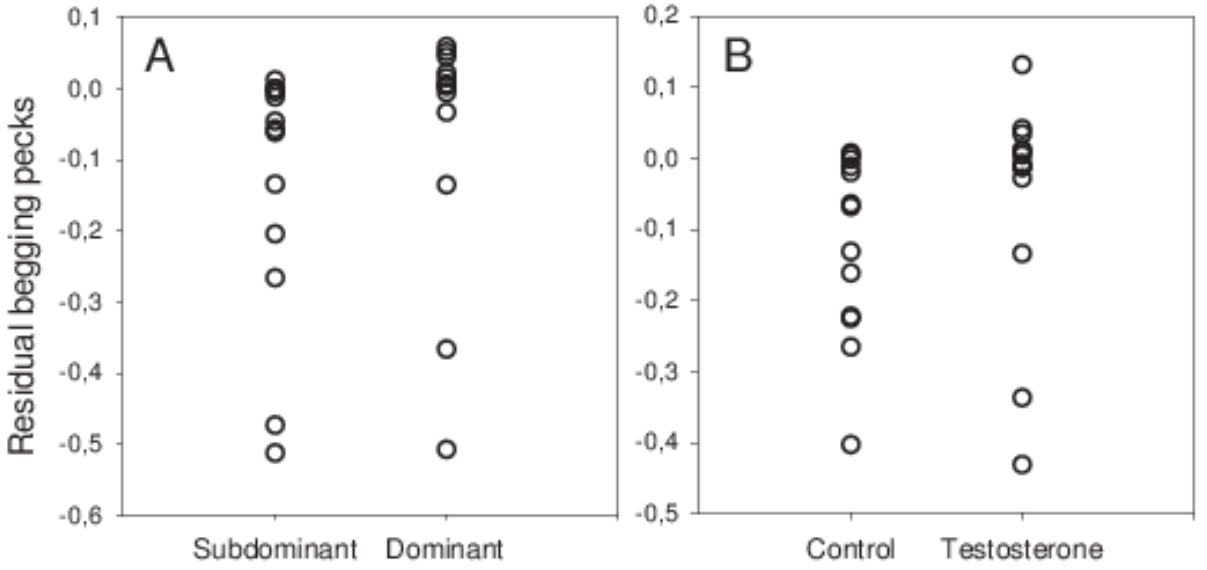

Figure 3: Residual total begging pecks toward parent, in relation to (A) dominance status, and (B) testosterone treatment. In each figure, the residuals were produced from a Poisson mixed model containing the predictors: dominance status, testosterone treatment, residual body mass, size differences between sibling pairs, size of the focal chick, and sex, minus the predictor represented on the x-axis. Both models contained also sibling dyad and observation number as a random factor.

Parameter	Estimate	SE	p-value
A. Both stimuli			
T treatment	1.83	0.52	**<0.001**
Stimulus type	-2.13	0.48	**<0.001**
Residual body mass	-0.01	0.009	0.33
Sex	0.51	0.52	0.33
B. Both stimuli			
Stimulus type*T treatment	0.01	0.95	0.99
Stimulus type*Residual body mass	0.03	0.02	**0.04**
T treatment	1.80	0.63	**0.004**
Stimulus type	-2.06	0.69	**0.003**
Residual body mass	-0.03	0.01	**0.03**
Sex	0.42	0.51	0.41
C. Dummy chick:			
T treatment	1.67	0.69	**0.02**
Residual body mass	-0.03	0.01	**0.02**
Sex	-0.15	0.71	0.84
D. Predator:			
T treatment	2.03	0.7	**0.004**
Residual body mass	0.01	0.01	0.28
Sex	1.04	0.68	0.13

Table 1: Parameter estimates, their standard errors and the corresponding p-values derived from Poisson linear mixed effects regressions on the number of aggressive pecks performed by kittiwake chicks during standardized behavioural tests using a dummy chick and/or a simulated predator as a stimulus.

Discussion

In this study we provide the first experimental evidence that circulating T increases sibling aggression in avian young. Compared with control chicks, T-treated kittiwake chicks aggressively pecked their sibling (and a dummy sibling) with higher frequency.

We also found that relatively larger siblings performed more aggression than their smaller siblings did. Naturally-occurring size asymmetries between kittiwake siblings arise because first-laid eggs hatch one or more days before second-laid eggs, and first-hatching chicks grow substantially before their siblings hatch. Relatively larger size (also correlated with motor development) is the main factor determining dominant status in broods of siblicidal species, and only behaviourally dominant chicks show aggression (Drummond 2006). In our study, we created sibling pairs in which the relative size differences between T and control chicks approximated a zero-centred normal distribution: in many cases the chicks were similar in size but in a few cases either a T chick or a control chick was substantially larger than its sibling. The independent main effect of size advantage in predicting aggression, and the lack of a significant interaction between T treatment and relative size difference between siblings, indicates that despite our experimental T elevation, relatively larger body size remained a significant determinant of the ability to subjugate a sibling.

We also found that chicks with lower residual body mass performed more aggressive pecks toward their sibling than did chicks with higher residual body mass. In facultatively

siblicidal species such as the kittiwake, siblicidal aggression is food sensitive (reviewed in Drummond 2001, White et al. 2010) and occurs at a high intensity only when body mass in dominant chicks falls below a critical threshold, which causes them to shift from a state of tolerance for their sibling to a state of intolerance (Drummond & Garcia Chavelas 1989). We expect that intolerant dominant chicks perceive the presence of their sibling as a social challenge and up-regulate T, as occurs in the obligately siblicidal Nazca booby during siblicide (Ferree et al. 2004), and elevated T sensitizes chicks to the social cue that triggers siblicidal aggression. Surprisingly, in our study, residual body mass predicted aggression in the same way for T-treated birds as it did for control birds, which suggests that an additional signal related to body condition modulates aggression irrespective of actual T levels. A likely candidate is corticosterone which is elevated in kittiwake chicks in poor condition and increases begging (Kitaysky et al. 2001) and aggression (Kitaysky et al. 2003; M. Müller, unpublished data).

Testosterone has been implicated as a mechanism underlying several types of aggression in young birds including aggressive displays toward foreign chicks entering their territories to steal food, (Groothuis & Meeuwissen 1992; Ros et al. 2002; Groothuis & Ros 2005) and sibling aggression (Ferree et al. 2004). We show for the first time that T simultaneously increases nestling aggression in multiple contexts in the same species: not only did T increase sibling aggression, T chicks also reacted more aggressively toward a simulated predator than did control chicks. Previous work showed that kittiwake chicks reared in an environment with a high risk of nest predation have higher testosterone concentrations than do kittiwake chicks reared in an environment with a low risk of nest predation (Ibáñez-Alamo et al. 2011). Our results suggest that kittiwake chicks may elevate testosterone to facilitate defensive attacks against potential predators.

We also found that kittiwake chicks showed an overall higher amount of aggression toward a simulated predator than toward a dummy sibling. Although kittiwake nests are situated on cliffs which protects them from most mammalian predators, avian predators including gulls, skuas (Burger & Gochfeld 1984) and ravens (MacCarone 1992) frequently enter kittiwake breeding colonies and depredate chicks directly from their nests. Our findings suggest that danger of predation triggers a stronger aggressive response than a potential competitor for food does. We found that this anti-predator aggression did not differ among chicks in relation to residual body mass, whereas aggression directed toward a dummy sibling was higher in chicks with lower residual mass than in chicks with higher residual mass. This latter finding is congruent with the negative correlation between residual mass and aggression that we found in chicks sharing a nest with a live sibling, and suggests that chicks did indeed perceive the dummy chick as a sibling. This difference between the two contexts makes sense from a functional perspective: anti-predator aggression should trigger an acute response independently from body condition, while siblicidal aggression in facultative siblicidal species depends on food availability and thus on body condition (Drummond & Chavelas 1989).

Dominant chicks begged more than did subdominant chicks, as has been reported in previous studies of kittiwakes (Cullen 1957; Braun & Hunt 1983). Here we show for the first time that T treatment increased begging intensity independently of its effects on dominance. The only previous experimental study showing that T increases begging intensity was performed in an altricial songbird (Goodship & Buchanen 2007) in which chicks compete to be the most visually conspicuous stimulus to the parents and so receive a larger share of the food (Forbes 2007). Kittiwake chicks beg via frequent vertical movements of the head toward the parent attempting to peck the corner of the parent's bill to stimulate regurgitation (Cullen 1957; Kitaysky et al. 2001) and siblings compete with each other for access to the parent. While dominant chicks have a competitive advantage in such situations and T elevation promotes this aggression-mediated dominance, our findings indicate that T has separate effects in promoting begging behaviour that are also expressed in subdominant chicks. This suggests that competitive begging in kittiwakes constitutes another example of a T-mediated social challenge, separate from aggression. In contrast, black-headed gull chicks, in which T was elevated by means of similar implants, actually begged less than controls did (Groothuis & Ros 2005). Interestingly, it has been suggested that these gull food solicitation displays toward parents are cooperative

rather than competitive in nature and signal the overall hunger of the brood (Roulin 2002; Mathevon & Charrier 2004; Forbes 2007). In this species, the parent does not feed a specific chick, as in altricial species and in the kittiwake, but regurgitates food for the whole brood onto the ground followed by scramble competition among chicks for their individuals shares. This suggests that T might only be involved in begging when performed in a competitive context.

In conclusion, T facilitates both siblicidal aggression and anti-predator aggression in young chicks, with body condition influencing only the former. T also facilitates, together with T-induced dominance, competitive begging behaviour. Therefore, T production in the kittiwake and most likely other siblicidal species seems an important fitness mediator already early in life, outside the sexual context and not only in an overtly aggressive context. Independently of T, the chicks modulate aggression based on social cues, e.g. dominance status and size difference.

Acknowledgements

We gratefully thank Roelie Veenstra-Wiegman for molecular sexing, Bonnie de Vries and Ilse Weites for help with testosterone assays and Roald Hansen for extensive logistical support in Ekkeroy. We thank Kjell Einar Erikstad for integrating our work in the study colony with the ongoing seabird monitoring (SEAPOP), and we thank the County Governor of Finnmark for permission.

Chapter 6. For better or for worse: Androgen-mediated maternal effects depend on the environmental context

Unpublished manuscript

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Abstract

Many plant and animal species use maternal effects to help downsize the number of propagules within a reproductive attempt if the resources available are insufficient for all young to grow into high quality progeny. In many birds, mothers begin incubating eggs before completing the clutch which causes hatching asynchrony among siblings, creating a size and developmental disadvantage in late-hatching (marginal) chicks. This size asymmetry among siblings facilitates rapid brood reduction via starvation of the marginal chicks when resources are too scarce to rear the entire brood. However, in the past two decades avian mothers were discovered to deposit relatively large amounts of testosterone into late-hatching eggs, which boosts their competitive abilities and has been hypothesized to mitigate brood reduction. This raised the paradox of why should mothers create hatching asynchrony and then counteract its effects via maternal testosterone? A possible solution to this paradox is that the benefits of maternal testosterone, (boosting competitive ability), outweigh its costs (increase of energy expenditure and suppression of immunity) in good food conditions, but that these costs outweigh the benefits in poor food conditions. We tested this in rock pigeons by manipulating both maternal testosterone exposure and food conditions. We found that in good food conditions, testosterone chicks grew better but in poor food conditions died more often than control chicks. Under good food conditions, testosterone chicks showed a weaker response to an immune challenge than did control chicks, and under poor food conditions, all chicks showed a weak immune response. This provides the first evidence for context-dependent hormone-mediated maternal effects, potentially explaining its adaptive co-evolution with the maternal effect generated by incubation-generated hatching asynchrony. It also might explain inconsistent results of egg hormone manipulations in the literature.

Introduction

Maternal effects occur when the phenotypes of offspring are influenced by the phenotype of their mother via non-genetic pathways (Mousseau and Fox 1998). They operate via diverse mechanisms that can influence developmental processes in the offspring as early as embryonic development and can increase the fitness of the mother and her offspring (Mousseau and Fox 1998; Marshall and Uller 2007). To date, most studies of the adaptive nature of maternal effects have focused on the matching of offspring phenotypes to the local environment, which may enhance the survival and breeding prospects (i.e. quality) of these offspring (Marshall and Uller 2007). But, because resources are limited, mothers must trade off offspring quality with offspring quantity (Stearns 1992) and in both plants and animals, maternal effects have been shown to help solve this trade-off (Mock and Parker 1997). Mothers can maximize the quantity of good quality offspring they can successfully rear given the breeding conditions by overproducing offspring, creating competitive asymmetries among siblings and allowing sibling rivalry to downsize the brood to the maximum number that parents can afford to rear without compromising the quality of the offspring. The intensity and outcome of sibling rivalry is determined by the environmental context: when resources are more abundant, fewer or no offspring perish and parents rear a relatively large number of young, whereas when resources are scarce, enhanced sibling rivalry causes higher offspring mortality so parents do not have to divide the few resources over too many offspring.

Mothers facilitate efficient context-dependent sibling rivalry-mediated brood reduction by creating competitive asymmetries within broods via a variety of maternal effects. For example, mothers might provision propagules with varying quantities of resources, but also other maternal factors such as steroid hormones, which can influence their relative quality and/or competitive ability upon emergence from the egg, seed, or mother's womb (e.g. von Engelhardt and Groothuis 2011, Frank et al 2008). Furthermore, mothers can influence the timing at which they emerge from the egg or seed: earlier emergence confers competitive advantages of relatively larger size and more advanced development compared to later-emerging siblings. The interplay of these maternal effects in mediating sibling rivalry-mediated brood reduction has thus far been studied extensively in birds (Müller and Groothuis 2013), which are an ideal model because the embryo develops outside of the mother's body allows manipulation of egg components and manipulation of the initiation of embryonic development without interfering maternal physiology (Groothuis *et al.* 2005b).

One of the most well-studied maternal effects relevant to sibling rivalry and brood reduction concerns the variation in the timing at which birds initiate egg incubation, which marks the initiation of embryonic development. In the majority of avian species, female birds lay eggs at a rate of maximal one egg per day. Because females start incubating before the completion of the whole clutch, the later-laid eggs hatch later and out of synch with the early-laid eggs. This hatching asynchrony creates a size hierarchy among siblings: core chicks, which hatched from eggs that were already laid before the onset of incubation, are a few days older and therefore bigger and more competitive than marginal chicks, which hatched from later-laid eggs (Mock and Forbes 1995; Mock and Parker 1997). With this hierarchy of size and competitive ability, the final brood size can be optimized for the environmental context via targeted morality of marginal chicks when necessary (resource-tracking hypothesis: Lack 1947, 1954, 1968, reviewed in Sockman *et al.* 2006; Müller and Groothuis 2013). According to this hypothesis, in a good year, when resources are abundant and parents can afford to raise all chicks, marginal chicks can contribute extra reproductive value by surviving alongside the core chicks, but in a poor year when resources become insufficient for parents to rear the whole brood, redundant marginal chicks are quickly outcompeted and die of starvation.

In addition to creating hatching asynchrony, avian mothers provision egg yolks with high concentrations of maternal androgens, which vary systematically across the laying order within a clutch (reviewed in Groothuis *et al.* 2005b; Gil 2008; von Engelhardt and Groothuis 2011). Exposure to maternal androgens has significant organizing effects on development and behavior (Nelson 1995; Adkins-Regan 2005; Carere and Balthazart 2007, Groothuis and Schwabl 2008)

that tend to increase competitive ability, so the widely-observed within-clutch variation in yolk androgen concentrations might enhance or mitigate competitive asymmetries present within avian broods. In fact, the leading hypothesis to explain the systematic within-clutch variation of yolk androgen concentration is that these hormones are tools by which avian mothers fine-tune the effects of hatching asynchrony (Hatching Asynchrony Adjustment Hypothesis, HAAH, Groothuis *et al.* 2005b) in a manner that facilitates survival of the whole brood or reduces redundant marginal chicks. In many species, the concentrations of yolk androgens increase over the laying order (e.g. Schwabl 1993; Lipar and Ketterson 2000; Sockman and Schwabl 2000; Eising *et al.* 2001; Mazuc *et al.* 2003; Verboven *et al.* 2003; Poisbleau *et al.* 2011; reviewed in Groothuis *et al.* 2005b; Gil 2008; von Engelhardt and Groothuis 2011). The increasing pattern of yolk androgens were thought to enhance the competitive ability of marginal chicks and mitigate the effects of hatching asynchrony (e.g. boosting the development and growth, Schwabl 1996; Eising *et al.* 2001; Pilz *et al.* 2004; Navara *et al.* 2006; Müller *et al.* 2007; stimulating begging behavior, Eising and Groothuis 2003; Barnett *et al.* 2011; increasing aggressive behavior, Müller *et al.* 2012), although results are not consistent in the literature (reviewed in Groothuis *et al.* 2005b; Gil 2008; von Engelhardt and Groothuis 2011). On the other hand, the decreasing pattern over the laying sequence in some other species might facilitate brood reduction (e.g. Schwabl *et al.* 1997; reviewed in Müller and Groothuis 2013).

However, the HAAH raises the following problem: why would females create hatching asynchrony to facilitate sibling rivalry-mediated brood reduction, and then use maternal androgens to mitigate the effects of hatching asynchrony (Groothuis *et al.* 2005b)? One possible answer is that rather than being an adaptive maternal effect, in some species hatching asynchrony may be a costly side-effect of adaptive early onset of incubation that reduces the risk of predation or preserves egg viability (Viñuela 2000; Arnold 2011). This is unlikely because the bulk of the evidence indicates that while eliminating hatching asynchrony reduces brood reduction, it comes at a serious cost to offspring quality, undermining the total brood value. Also not much evidence exists for the egg viability hypothesis (for an extensive discussion see Groothuis et al 2005b).

A more likely resolution to the paradox created by the HAAH is that yolk androgens actually have different effects depending on the environmental context in such a way that it acts in concert with the context-dependent effects of hatching asynchrony, either facilitating brood survival or brood reduction (Müller and Groothuis 2013). Although exposure to yolk androgens has been shown to have numerous beneficial effects on development, growth, and competitive ability of offspring, prenatal exposure to elevated maternal androgens also carries costs. For example, in zebra finches (*Taeniopygia guttata*), the resting metabolic rate of chicks hatched from testosterone-injected eggs was significantly higher than in control chicks in both the nestling stage (14-15 days, Tobler *et al.* 2007) and the adult stage (5-7 months, Nilsson *et al.* 2011). Prenatal exposure to elevated androgen levels were also found to suppress immune function in Chinese painted quails, *Coturnix chinensis* (Andersson *et al.* 2004), black-headed gulls, *Larus ridibundus* (Groothuis *et al.* 2005a; Müller *et al.* 2005), eastern bluebirds, *Sialia sialis* (Navara *et al.* 2005), and jackdaws, *Corvus monedula* (Sandell *et al.* 2009). It is therefore conceivable that a different balance in costs vs. benefits of elevated maternal androgen exposure occurs depending on the environmental context (especially depending on food availability), inducing context-dependent effects in avian young. In a good year, when food resources are abundant, chicks might receive enough food to support their higher energy expenditure and immune system. But in a poor year, when the available resources are not sufficient to support these costs, the costs are likely to outweigh the benefits, causing high yolk androgen exposure to have negative rather than positive effects on survival. If this is the case, yolk androgens enhance the adaptive effects of hatching asynchrony in context-dependent brood reduction: in good environmental conditions, higher levels of yolk androgens increase the competitive ability of marginal chicks and boost their survival, mitigating the effects of hatching asynchrony, while in poor environmental conditions, higher levels of yolk androgens become detrimental and facilitate brood reduction, exacerbating the effect of hatching asynchrony (Müller and Groothuis 2013).

An obvious way to test for context-dependent effects of yolk androgens would be to combine a manipulation of yolk androgens and a manipulation of food availability during the parental care phase. To our knowledge, no such experiment has yet been conducted. The only one study to approach such an experiment is a study by Cucco *et al.* (2008), which injected different doses of testosterone into grey partridge (*Perdix perdix*) egg yolks and supplemented the diet with β-carotene in half of the chicks from each testosterone treatment. Although carotenoids are not the same thing as calories (and our hypothesis is based on energy limitation), they found an interaction effect between both treatments providing evidence that the effects of yolk androgen exposure differs depending on resources. Another study, more relevant to our hypothesis, (Pitala *et al.* 2009), manipulated androgen levels in the eggs of two different populations of collared flycatchers (*Ficedula albicollis*), breeding in an apparent rich and poor food environment and found that effects of yolk androgens did not correlate with different environments.

In this study, we tested whether effects of yolk testosterone differ depending on the environmental context, in an experiment on rock pigeons (*Columba livia*) that combined *in ovo* testosterone injections in freshly laid eggs, and food restriction treatment after chicks hatch. The modal clutch size of rock pigeons is two (Johnston and Janiga 1995), and the second egg always contains a substantially higher amount of testosterone in the yolk (see method section; V. Goerlich *et al.* unpublished data; B.Y. Hsu *et al.* unpublished data.). In this study, we used only first-laid eggs, containing low levels of maternal testosterone, from different (but synchronous) pairs to create new experimental clutches that contained one egg that was injected with testosterone solution to the level of second eggs, and the other one that was only injected with vehicle as control. Half of these experimental clutches produced broods that were reared in experimentally-imposed poor food conditions while the other half were raised in a good food conditions. To avoid that mothers adjusted egg composition to the food situation, food manipulation was initiated only from the time of hatching onwards. Over the course of the experiment, we monitored various aspects of growth and development as well as immune function. We expected to see beneficial effects of elevated yolk testosterone levels on chick growth and immune function under the good food condition, and detrimental effects, especially enhanced early mortality of chicks, in the poor food condition.

Materials and Methods

Housing

The pigeons used in this study came from our colony housed in a large outdoor aviary (45m long x 9.6m wide x 3.75m high). In early April 2012 they were re-housed in several smaller identical aviaries (4.01m long x 1.67m wide x 2.2 m high) in the animal facility of the Centre for Behaviour and Neurosciences of the University of Groningen. Two pairs of pigeons were housed in each of the smaller aviaries. Because the pair bonds among feral pigeons can last for their whole lifetime (Johnston and Janiga 1995), we selected already established pairs when known, or randomly-paired males with females if their partner identity was unknown. Established and random breeding pairs were assigned to the two food condition treatment groups in an equal proportion. After re-housing them in the smaller aviaries, pigeons were given at least one week to acclimate before the experiment started.

Each aviary was provided with standard food for pigeons (KASPER TM 6712), and for Streptopelia (KASPAR TM 6721, see Table E in the appendix for specifications), water, and a mixture of small stones and pigeon grit *ad libitum*, until the food treatment started. To initiate breeding, two nest boxes (60 cm long*50 cm deep*36 cm high) with nest materials and a nest bowl were placed into each aviary.

All experimental and animal care procedures were under the approval of the animal welfare committee of University of Groningen (DEC No. 5635D) and complied with Dutch law.

Egg collection, incubation, hatching time and the creation of experimental broods

This experiment was conducted from May until September of 2012. In early May, we opened the doors of the nest boxes to induce breeding. Nest boxes were checked every morning and all freshly-laid eggs were collected from nests, marked by permanent markers, and replaced with a dummy egg so parents would continue incubating. We only used first-laid eggs (A-eggs) for the experiment and all second-laid eggs (B-eggs) were cross-fostered to the colony when possible. Collected A-eggs were stored in a climate cell with relatively constant temperature (12-16°C) and humidity (40-50%) for no more than three days, until a large enough batch of eggs had been collected for allocating eggs to experimental pairs. We paired A-eggs by matching laying date and mass as closely as possible, and then injected one egg in each pair with either testosterone solution (T-eggs, see below for dosage) or sesame oil (C-eggs). After injection, these eggs were immediately returned to an unrelated foster nest for incubation. Between T- and C-eggs, there were no significant differences in mass (T-eggs, 17.00 ± 0.13 g, n = 95; C-eggs, 17.07 ± 0.14 g, n = 95; t = 0.383, p = 0.7021) and laying date (Mann-Whitney U test, p = 0.9779).

In order to record the hatching time as accurately as possible, on day 16 of incubation, all eggs were again replaced by dummy eggs and placed into an incubator until hatching. The incubator was maintained at constant 37.5 °C and humidity was kept higher than 75%. On the same day that eggs were placed in the incubator, we randomly assigned each aviary to rich food conditions or poor food conditions (for specifications see below) and started the experimental food treatment.

The incubator was checked three times during the daytime (9:00, 13:00, 17:00). If obvious signs of hatching (e.g. a large hole through which the bill of the chick can be seen, or a large crack) were observed at 17:00, the incubator was checked again at 21:00. Hatchlings found in the incubator at 13:00, 17:00 and 21:00 were recorded as having hatched in the morning, afternoon, and evening. Hatchlings found at 9:00 were identified has having hatched at midnight or at dawn depending on the wetness of their down.

For each hatchling, the body mass, head-bill length, tarsus length, and wing length were measured (see below), and a small blood sample (< 75µl) was taken from the medial metatarsal vein for sexing. Chicks of opposite treatments (T- or C-injection) were paired in a way that matched body mass and hatching time as closely as possible and were returned to a foster nest. Within the pair that were returned to the same nest, T-chicks hatched slightly earlier but this was not statistically significant (n = 29, t = -1.79, p = 0.084); body mass did not differ between hormone treatments (t = -1.229, p = 0.229). Pigeons have slight but significant sexual size dimorphism (Janiga and Johnston 1995). In order to increase the sensitivity of our experimental design, chicks were swapped to increase the number of sex-matched T-C paired broods wherever possible within two days post-hatching, directly after the sex was determined. but only when they had similar body masses. In a few cases we had to swap chicks among food treatments (n = 3 pairs) assuming that the effect of treatment so early after hatching, when chicks do not demand much food and partly still consume yolk, was not important.

Egg injections

The testosterone levels in the yolks of experimental eggs were manipulated by *in ovo* injection. Either 50 ml T solution or pure sesame oil was injected into the egg yolk using a 0.5 ml U-100 insulin syringe (0.33mm(29G) needle×12.7mm syringe, BD Micro-Fine ™). We dissolved crystalline testosterone (Fluka) in sterilized sesame oil at a concentration of 920 ng T/ml oil, so that injecting 50 ul of this solution elevated the amount of testosterone in these A-egg yolks (mean T = 23 ng T per yolk) to the amount present in B-eggs (mean T = 69 ng T per yolk, V. Goerlich et al, unpublished). Before this study started, we again measured T in 11 un-manipulated clutches of eggs to check this difference between A and B eggs for our colony in the year we carried out our experiment. The mean difference of T per yolk between A and B eggs was 44.99 ± 10.38 ng, which was not significantly different from 46 ng (one-sample t-test, t = -0.1061, p = 0.9176). During injection, eggs were placed on their side for a few minutes, allowing the yolk to float up. Then the eggshell was punctured from the blunt end by a needle attached to

an insulin syringe filled with T solution or sesame oil. After withdrawing the syringe, the hole in the eggshell was sealed by a small piece (~25mm^2) of artificial skin (Hansaplast™).

Food treatment

About two days before chicks hatched, food treatment started. Pigeons in the good food conditions treatment group were provided with the same grain mixtures as in the standard food condition plus *ad libitum* pigeon pellet (KASPAR ™ P40, for specifications see Table E in the appendix) and supplemented with vitamin powder (Supralith ™). Pigeons in poor food conditions were fed with 33g "chicken" grain mixture per pair per day, which is what a pair of adult pigeons consumes in a day based on our previous measurements. In order to accommodate increased energy demands as chicks grew, we provided the food-restricted group with additional food when chicks were older than one week, based on Jacquin *et al.* 2012. For every 7-day-old or older chick, we gave 8g additional food; for every 14-day-old or older chick, 16g additional food was provided.

After the experiment, the percentage of body mass lost in adult pigeons in poor food conditions was significantly higher than adults in good food conditions (good food condition, -6.31 ± 0.75, n = 67, poor food condition, -13.77 ± 1.16, $t = 5.395$, $p<0.0001$), indicating that our experimental food restriction significantly reduced energy available to breeding adults.

Molecular sexing

Our molecular sexing procedure followed the protocol described in Goerlich *et al.* (2009, 2010). The DNA of pigeon chicks was extracted with Chelex (Walsh *et al.* 1991) from blood samples stored in 70% ethanol. Polymerase Chain Reaction (PCR) with primers P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTCCCAAGGATGAGRAAYTG-3') were used to amplify the two homologous genes CHD-W (females) and CHD-Z (males and females) on the sex chromosomes (Griffiths *et al.* 1998). For every PCR and gel electrophoresis, control samples of a known female (two bands) and male (one band) were also included. Chicks were sexed within the first 2-3 days after hatching so that same-sex pairs could be created early in the nestling period.

SRBC test and haemagglutination assay

We used sheep red blood cells (SRBC) as antigens to test aspects of immunocompetence of pigeon fledglings. This method has been long used to induce a humoral immune response in avian species (e.g. Deerenberg *et al.* 1997; Ros *et al.* 1997, Hanssen et al 2004, Rutkowska et al. 2012). All SRBC used in this study were from one identical sheep and stored in Alsever's solution (S.B.-0011, Harlan ™). New SRBC in Alsever's solution was washed with phosphate buffered saline (PBS) and diluted to 2% SRBC solution in PBS (5×10^8 cells/ml). When chicks were about one month old and maternal antibodies were no longer present (Hasselquist and Nilsson 2009), every pigeon fledgling was immunized with 0.5 ml 2% SRBC solution via intraperitoneal injection. Two days prior to injection, a blood sample (100-200 µl) was taken from the brachial vein (0d samples). Six days after SRBC injection, at the day of peak antibody production, (see for example Ros *et al.* 1997, Casagrande et al. 2012), another blood sample was taken. Both blood samples were centrifuged and the plasma and blood cells were separated. The plasma samples were stored at -20°C for later haemagglutination assay to estimate the circulating anti-SRBC antibody levels.

The protocol of haemagglutination assay was based on Ros *et al*. (1997). The frozen plasma samples were thawed under room temperature and place in a water bath at 56°C for 30 min to remove proteins that will lyse the sheep red blood cells. Then 20 µl of the plasma was taken and diluted 1:1 in PBS in the first column of a U-shaped microtiter plates, and further diluted in a series of 2^2, 2^3, 2^4… to 2^{12}. Twenty µl freshly-washed 2% SRBC solution was added to every well and all titer plates with samples were incubated at 37°C for one hour. Based on the

presence of haemagglutination, the antibody titers were scored visually as the highest dilution of plasma by B. Riedstra, who was blind to all treatments on pigeons. The scores were then represented as integers on a $\log_2$ scale. If the plasma was enough, we did a second duplicate and took the mean score from the two duplicates of the plasma sample.

Growth monitoring and growth models

The growth of chicks was monitored by measuring body mass, head-bill length, tarsus length, and wing length every two days in the first two weeks after hatching, and every three days from two weeks to 26 days post-hatching. Body mass was measured using a digital scale with an accuracy of 1g. Head-bill length and tarsus length were measured with digital calipers to the nearest 0.01 mm. Head-bill length was measured from the back of the skull to the tip of the bill. We also measured the length of the tarsometatarsal bone on the right leg ("tarsus length"). Wing length was measured from the carpus to the tip of the skin (when chicks did not have primaries) with a digital caliper to the closest 0.01 mm or to the tip of the longest primary feathers with a ruler to the closest 0.5 mm. All wing length data were measured from the right wing. All body measurements were taken by the same experimenter (BYH).

After taking the 6d samples of SRBC test (for most chicks, 37 days after hatching, about one week after fledging, Johnston and Janiga 1995), chicks in good food condition were all able to feed themselves and were separated from their parents. Our food restriction in poor food condition delayed the age of independence; therefore we did not separate them from parents until they were observed to be self-sufficient in pecking seeds.

Sample sizes and data analyses

We injected 190 eggs in total (95 T-eggs and 95 C-eggs) and 93 eggs hatched successfully (48.95%). Among the successfully-hatched eggs, 44 were C-eggs and 49 were T-eggs. For 5 out of the 93 successfully-hatched chicks, we were unable to create proper experimental pairs, so they were excluded from the experiment. Out of the remaining 88 chicks we could create 34 T-C pairs matched for body mass and hatching date. Only 19 of these pairs (10 pairs in good food condition and 9 pairs in poor food condition) were also matched for sex and thus included in the analysis for the interaction between hormone injection and food treatment.

All statistical analyses were performed with the software R 2.13.2 (R Development Core Team 2011). We analyzed hatching success using a logistic regression, with hormone injection as the independent variable. To control for possible effects of season and egg mass, we included date of return to the foster nest and egg mass as covariates.

We regarded those eggs that were injected and returned to the foster nests on the same day as coming from the same batch. Within each batch, the hatching time of each egg was ranked and then we used the Mann-Whitney U test to test the effects of hormone injection on hatching time.

To analyze hatchling body mass, we used linear regression, including hormone treatment, egg mass, sex, and all interaction terms as independent variables. The full model was then backward-selected using a step-wise procedure via the function *stepAIC* in the library MASS (Venables and Ripley 2002) to determine the final model.

Because the sample size for T-chicks in poor food conditions after day 8 decreased to a low value due to the high mortality in this group (see Results), yielding a very low statistical power, we analyzed the effects of the treatments on body mass at day 8, just before mortality substantially increased, with the same model as above, but adding food treatment and its interaction as additional predictors. We used linear regression for this analysis because data of body mass at day 0 and 8 were normally distributed (Shapiro-Wilks test, p values > 0.25).

Because of the high mortality of T-chicks in poor food conditions, we could only analyse the effect of the hormone treatment on growth curves for chicks of control and hormone injected eggs in the good food condition. Because the growth data of the four biometry variables were all

sigmoidal, we chose two most commonly used models in bird species, the Gompertz growth model and the logistic growth model, to build the estimated growth curves of chicks:

Gompertz growth model: f(t)=Asym*exp(-b2*b3^t)

Asym, b2, and b3 represent the asymptote, initial value, and the inflection point of a biometry variable, respectively, and b2 and b3 both also influence the growth rate constant (Sockman *et al.* 2008).

Logistic growth model: f(t)=Asym/1+exp((xmid-t)/scal)

Asym represents the asymptote of a biometry variable; xmid represents its inflection point, and scal is the inverse of the growth rate constant (Tjørve and Tjørve 2010).

We used the self-starting functions *SSgompertz* and *SSlogis* in the package *stats* of R (R Development Core Team 2011) to fit the individual growth curves. Akaike Information Criterion (AIC) was used to compare the goodness of fit between two growth models and determine which one would be used for the follow-up non-linear analysis. For body mass, Gompertz model proved to be a better fit, while for the other three biometry variables, logistical growth model proved to be a better fit.

The four biometry variables were analyzed separately, and only the data from broods in which two chicks were sex-matched and hatched on the same day were used in the analysis of growth. We used non-linear mixed-effects models by R package *nlme* (Pinheiro *et al.* 2011) to examine which factor had effects on the three parameters of the growth curves. Based on the procedures of Sockman *et al.* (2008), the non-linear mixed-effects model of our data contains two levels: level 1 describes how a biometrical variable changes over time, and level 2 shows which factor might influence the variation across individuals. At level 1, we used separate growth models (Gompertz equation for body mass and logistic equation for head-bill, tarsus, and wing length) to describe the growth of every individual bird. At level 2, the three parameters of the growth model at level 1 were considered as the dependent variables. The treatment of *in ovo* injection, the sex of the chick, and their interaction were included as independent variables. Egg mass was also included as a covariate. These predictors formed a linear function for every parameter of the growth model (Asym, b2, b3 in Gompertz equation or Asym, xmid, scal in logistic equation) at level 1, which allowed us to examine the effects of all predictors on every parameter of the growth curve by linear regression.

Level 1 and level 2 models both included fixed and random effects. At level 1, the fixed effects were the growth equations, and we used chick identity as the random effects. At level 2, the fixed effects were the aforementioned predictors for the three parameters of the growth equations. Initially, the random effects at level 2 contained the identical three parameters initially as the fixed-effects but did not contain any predictor. Following the recommendation of Pinheiro and Bates (2000), we tested whether all parameters of the random effects at level 2 were necessary and left out the unnecessary one(s) from the model to avoid over-parameterization. If the variance of residuals showed a systematic pattern, like increasing across fitted values, we specified the relevant variance function in the model as Pinheiro and Bates (2000) suggested, and further confirmed the goodness of fit by AIC.

All the predictors we considered might influence every parameter of the growth curve independently and we could not apply a normal backward or forward step-wise model selection approach. Therefore, using AIC (Burnham and Anderson 2002) as a criterion, we first used a forward approach to determine the best-favoured model for each parameter separately. Then we combined the best-favoured models and further sought the possibility of simplification by leaving out one factor in a step-wise fashion. Then AIC was again used as a criterion to determine the final model (for all models we compared in our model selection strategy, see Appendix).

For the analyses of survival we recorded the age of fledglings on the day that we separate a fledgling from its parents and used these as the maximum ages of surviving chicks in

the survival analysis. The survival data were therefore right-censored, because for these surviving chicks we did not know their time of death in the future. R package *OIsurv* (Diez 2012) was used for survival analysis. The survival curves were built for T- and C-chicks in each food condition, and the Gehan-Wilcoxon test with Peto & Peto modification (rho = 1) was used to test the differences of survival curves.

For our analyses of immunocompetence, the final scores derived from SRBC and haemagglutination test were calculated by subtracting the score of the day 0 sample from the score of a 6d sample from the same chick. Normal distribution of the data was confirmed by Kolmogorov-Smirov tests and we applied a linear model with hormone treatment on eggs, sex of the fledgling, food condition, and all interaction terms. Chick age at SRBC injection was also included as a covariate. The full model was then backward-selected using a step-wise procedure via the function *stepAIC* in the library MASS (Venables and Ripley 2002) to determine the final model. When the final model included interactions, we performed an additional model excluding the interaction so that we could interpret the main effects.

Results

Before start of food treatment: Effects of testosterone treatment on hatching success, hatching time, and hatchling body mass

In the logistic regression, neither T treatment nor season significantly influenced hatching success (n=190, p=0.407 and 0.861 resp.). Heavier eggs were more likely to hatch successfully (estimate = 0.339, s.e. = 0.117, z = 2.899, p=0.0037). There was no difference in time of hatching between T- and C eggs (Mann-Whitney U test, W=1206.5, p=0.3114), with the majority in both groups hatching at night or dawn (n=29 and 28, respectively).

The final model of hatchling body mass (selected by *stepAIC*) did not include T treatment in the model as this was not significant (estimate = 2.104, s.e. = 3.028, t = 0.695, p = 0.489). The model retained only sex, egg mass and their interaction, with only the latter two being significant (interaction: estimate = -0.253, s.e. = 0.118, t = -2.138, p = 0.0354; main effect egg mass: estimate = 0.612, s.e. = 0.06, t = 10.134, p < 0.0001; main effect sex: estimate = -0.037, s.e. = 0.157, t = -0.234, p = 0.816). The interaction suggested that males are heavier in relation to light eggs but post hoc tests did not convincingly support this (p values > 0.09). In a separate model in which we excluded the interaction to test the significance of main effects, we found a positive effect of egg mass on hatchling mass (estimate = 0.612, s.e. = 0.06, t = 10.134, p <0.001) but sex was not a significant predictor (estimate = -0.037, s.e. = 0.157, t = -0.234, p = 0.816).

After the start of food treatment: Chick survival

The survival of T- and C-chicks differed in the two food conditions. In good food conditions, chick survival was 100%, regardless of hormone treatment. In poor food conditions, T-chicks had significantly lower survival than C-chicks (Gehan-Wilcoxon test, n=18, p=0.0489, Fig. 1). The interaction effect of food treatment and T injection was highly significant (Gehan-Wilcoxon test, n=38, p < 0.001, Fig. 1).

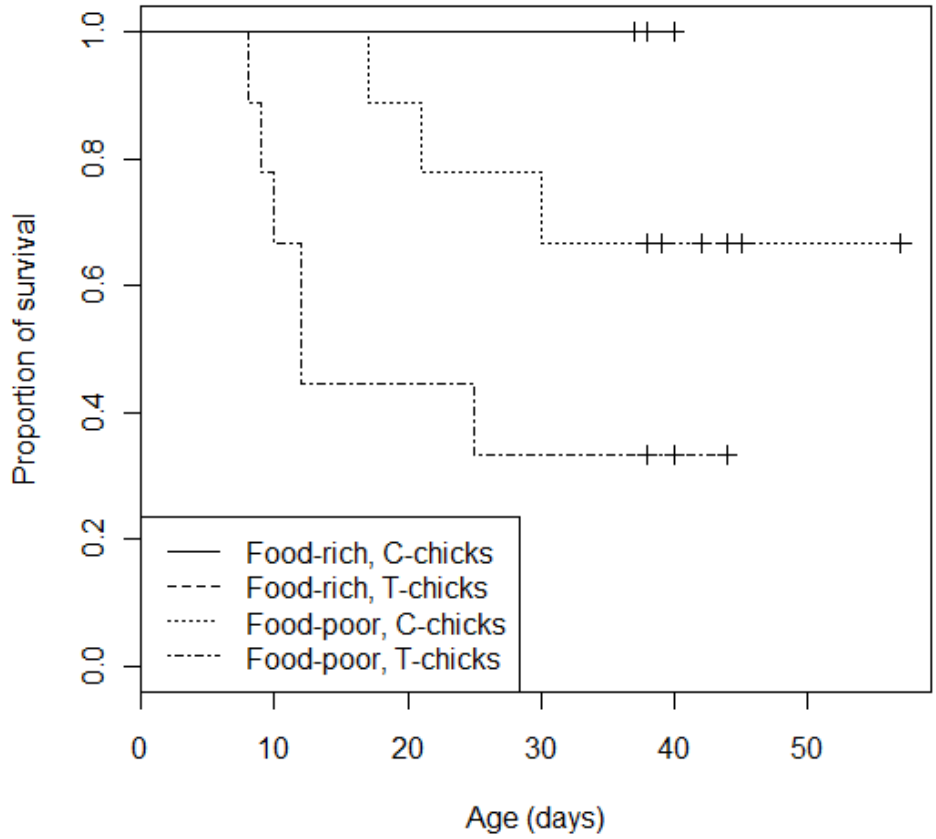

Figure 1. Survival curves of T-and C-chicks in good and poor food conditions. In good food conditions, chicks from both egg injection treatment had 100% survival so curves for T- and C-chicks overlap completely. In poor food conditions, T chicks had lower survival than C chicks (n=18, p=0.0489), and the interaction between food treatment and T treatment over all chicks was significant (n=38, p=0.001).

After the start of food treatment: Body mass at day 8 after hatching

The final model retained T treatment, food treatment, their interaction, and egg mass. In this model, egg mass was positively correlated with body mass (estimate = 5.382, SE = 1.758, t = 3.061, p = 0.0044). The interaction effect of T treatment and food treatment was significant (Fig. 2, interaction: estimate = -19.476, SE = 8.896, t = -2.189, p = 0.03575, main effects of T treatment: estimate = 16.800, SE = 6.086, t = 2.761, p = 0.0094 and food treatment: estimate = -44.684, SE = 6.253, t = -7.146, p < 0.0001). A post hoc Tukey HSD test showed that T-chicks tended to be heavier than C-chicks in good food conditions (p=0.077), but that there was no difference in body mass between T- and C- chicks in poor food conditions (p=0.925). When we performed this model without the interaction so that we could interpret main effects, we found that chicks reared in poor food conditions were much lighter (estimate = -54.323, s.e. = 4.681, t = -11.605, p<0.0001), chicks hatched from heavier eggs were heavier (estimate = 5.805, s.e. = 1.842, t = 3.151, p=0.0034), and T treatment did not show an overall significant effect (estimate = 7.686, s.e. = 4.680, t = 1.642, p = 0.11).

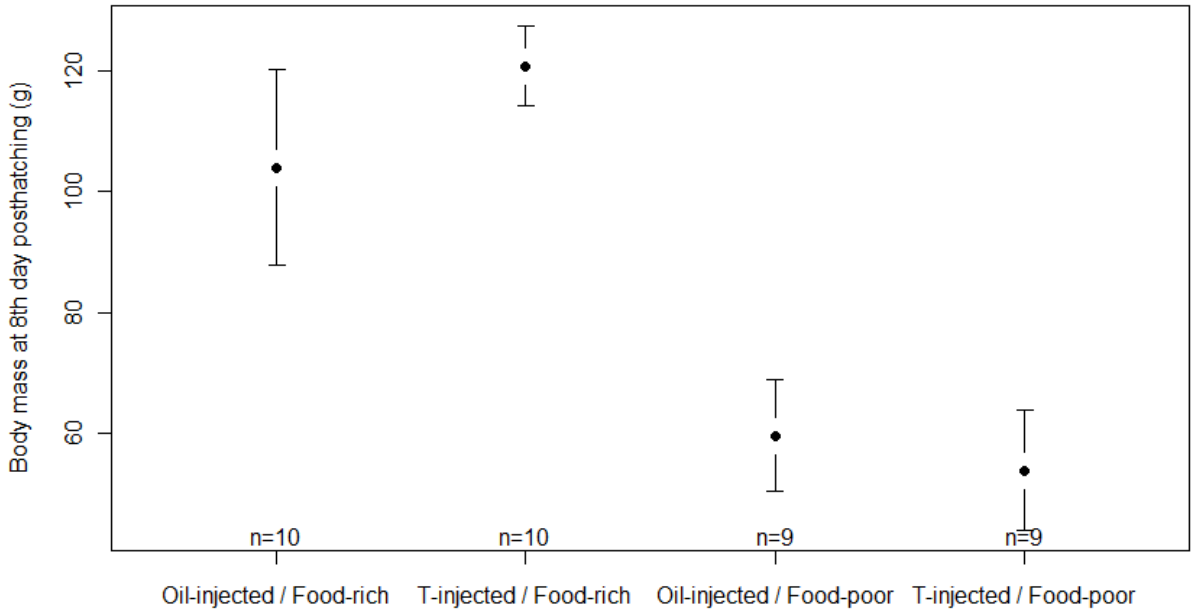

Figure 2. Body mass of chicks at day 8 after hatching (interaction of food treatment and T treatment, p=0.0358).

After the start of food treatment: Growth

The best final models for the four morphometrical data sets (body mass, head-bill length, tarsuslength, and wing length) indicate that hormone treatment of the egg affected growth in terms of body mass (marginally significant, p= 0.053), with T-chicks reaching the inflection point earlier than C-chicks (tables 1-4: "b3.T injection"). For all four measurements, sex was retained in the model, significantly affecting the asymptote (tables 1-4: "asymp.sex"), with males being heavier and larger than females, although this did not reach significance for head-bill length. Finally, egg mass positively and significantly all four growth measurements, positively affecting initial body mass (table 1: "b2.egg mass"), the inflection point of the other three measures (tables 2-4: "xmid.egg mass"), the asymptote of tarsus length (table 3: "asym.eggmass") and the growth rate constant of head-bill length (table 2: "scal.eggmass").

After the start of food treatment: Immunocompetence

The final linear regression model retained T treatment, food treatment, sex, and the interaction between T treatment and food treatment. Sex was a significant predictor of immune response (estimate = -1.899, SE = 0.670, t = -2.836, p = 0.006, n = 66) with male chicks producing fewer antibodies than females chicks (p=0.005; antibody scores of males: 2.93±3.25, n=34 and females: 4.39±2.95, n=32). In this model, having very few surviving T-chicks in the data set, the interaction effect of T treatment and food treatment was not statistically significant (estimate = 2.141, SE = 1.506, t = 1.422, p = 0.160, main effect of T treatment: estimate = -2.349, SE = 0.775, t = -3.029, p = 0.004, main effect of food treatment: estimate = -4.004, SE = 0.966, t = -4.143, p < 0.001). In a model that excluded the interaction, chicks reared in poor food condition produced fewer antibodies in response to the sheep red blood cell challenge than did chicks reared in good food conditions (estimate = -3.127, SE = 0.750, t = -4.169, p < 0.0001, Fig. 4), and T-chicks showed a lower immune response than did control chicks (estimate = -1.794, SE = 0.676, t = -

2.655, p = 0.01, Fig.4). In this model sex was still significant (estimate = -1.957, SE = 0.674, t = -2.905, p = 0.005).

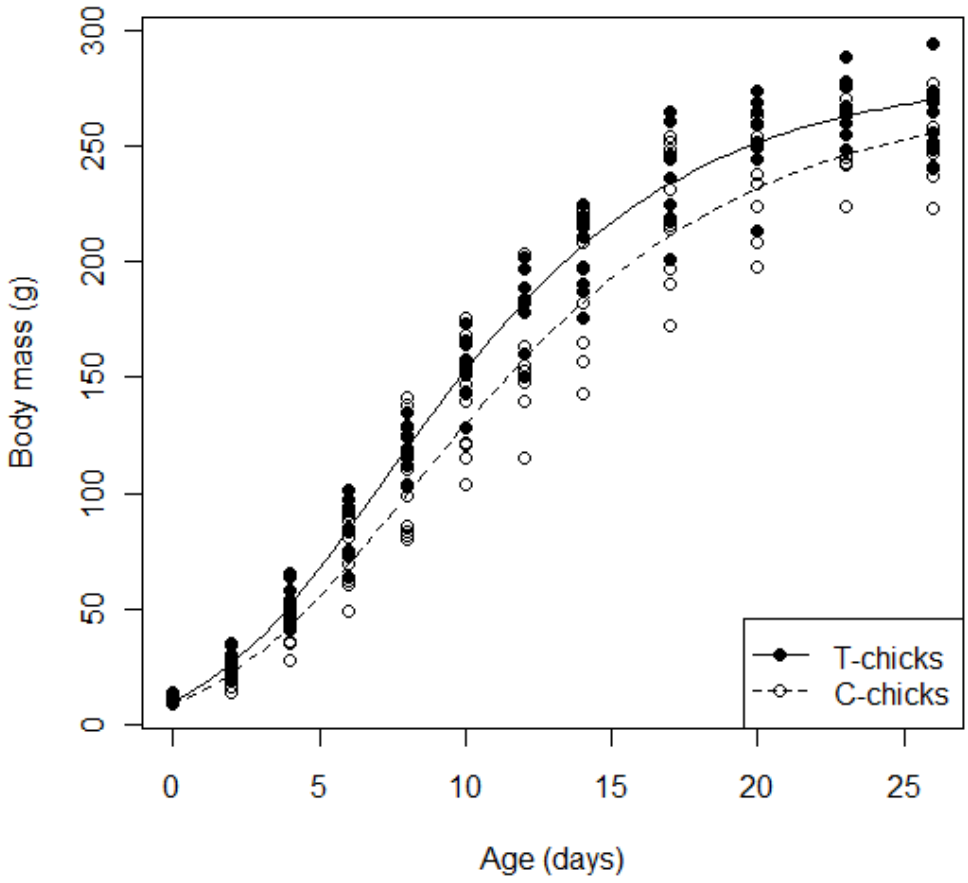

Figure 3. Growth curves of body mass (non-linear mixed-effects model with Gompertz growth curve) for T and C birds in good food condition.

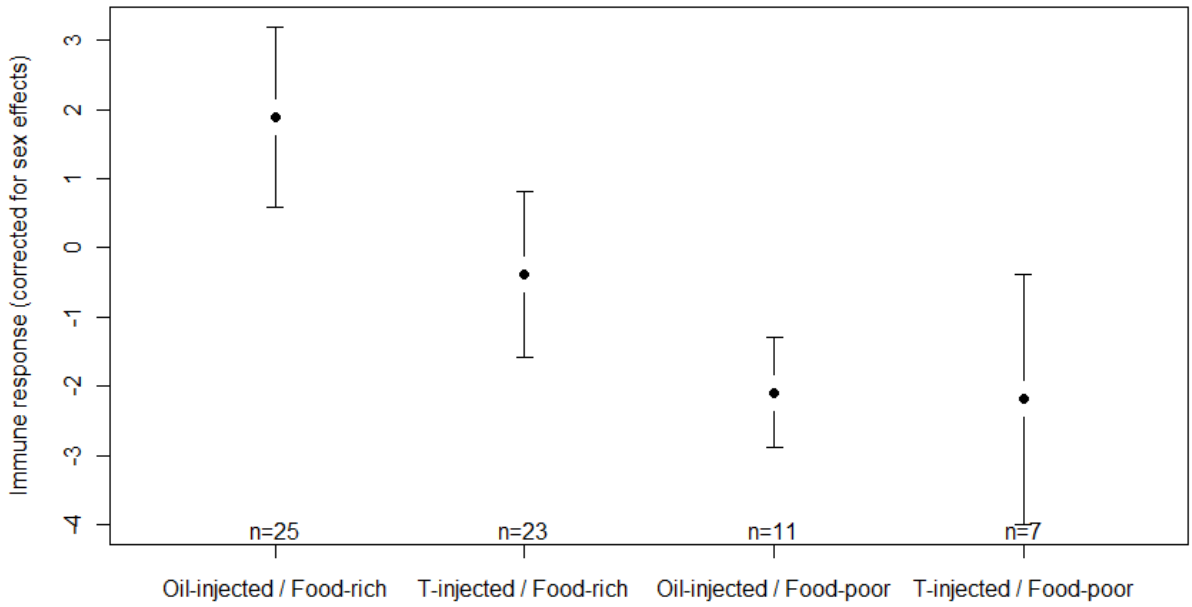

Figure 4. Results of immune challenge (sheep red blood cell test and haemagglutination assay).

Discussion

In many species mothers favour some progeny over others and often adjust offspring numbers to prevailing food conditions by creating core and marginal young, of which the former survive at the cost of the latter under poor food conditions. Birds, which create hatching asynchrony by their incubation pattern, are the best-studied example of this. The detection of substantial amounts of maternal androgens in egg yolks that systematically vary with laying order and hatching asynchrony, has added additional layer of complexity. The hatching asynchrony adjustment hypothesis (HAAH) states that mothers produce clutches with increasing concentrations of maternal androgens over the laying sequence to counteract the detrimental effects of late-hatching in last-laid eggs (Schwabl 1993, 1996, Groothuis et al. 2005b) and support the survival of the whole brood. This created the following paradox: why would avian mothers create hatching asynchrony to disadvantage late-hatching chicks and then provide them with extra yolk androgens to counteract this effect? Our findings provide a solution to this paradox. We found that exposure to elevated yolk androgens did indeed benefit late-hatching (marginal) nestlings under food-rich conditions by enhancing growth (fig. 2,3), but caused higher mortality when food conditions were poor (fig. 1). This is the first experimental evidence for context-dependent effects of maternal hormones. Here we place within-clutch patterns of maternal androgens into a different framework, stating that context-dependent effects of yolk androgens work in coordination with the context-dependent effects of hatching asynchrony in supporting adaptive brood survival in good breeding conditions and adaptive brood reduction in poor breeding conditions (as suggested by Müller and Groothuis 2013). Therefore maternally-engineered competitive asymmetries within broods arising from variation in yolk androgens and hatching asynchrony and allow parents to maximize the number of offspring they rear without compromising their quality.

Many of our individual results fall in line with previous research. For example, several other studies have also demonstrated beneficial effects of experimentally-elevated yolk androgens on the early growth of nestlings (Schwabl 1996; Eising et al. 2001; Pilz et al. 2004; Groothuis et al. 2005a; Navara et al. 2006; Müller et al. 2007). Furthermore, similar to our findings, several other studies have found that exposure to elevated yolk androgens can reduce survival (Sockman and Schwabl 2000; Müller et al. 2007, 2009). But we show for the first time, within the same study that the direction of the effects of maternal androgens depend on prevailing food conditions during the chick-rearing period.

An increase in growth in chicks exposed to elevated levels of yolk testosterone might be due an increase in begging behaviour, as has been found in several studies (e.g. Schwabl 1996, Eising and Groothuis 2003), which would result in higher food delivery by the parents. What remains unresolved is the mechanism by which elevated yolk testosterone induced detrimental effects, especially the strong increase in mortality in poor conditions. In our study, most nestling mortality occurred after 8 days of age. The energy and nutrient demands of chicks most likely peaked around 10 days after hatching because the inflection point of the growth curve, at which the growth rate reaches its maximum, occurs around 10 days (Fig. 3). This period of high resource allocation toward growth was even more pronounced in T-chicks (as observed in larger mass in T-chicks at day 8 and a marginally significant earlier inflection point in the body mass growth curve under good conditions). Together with higher energy requirements due to the fact that yolk testosterone increases metabolic rate (Tobler et al 2007, Nilsson et al 2011) and begging behaviour (Schwabl 1996, Eising and Groothuis 2003, von Engelhardt et al 2006) these higher requirements for energy may have had fatal effects on the energy balance of chicks from T-treated eggs in poor food condition.

T-chicks also showed a weaker response to an immune challenge (injected sheep red blood cells), consistent with several findings in other avian species (Andersson *et al.* 2004; Groothuis *et al.* 2005a; Müller *et al.* 2005; Navara and Mendonca 2005; Sandell *et al.* 2009). As all fledglings reared in poor food conditions showed a weak response to an immune challenge compared to fledglings in good food conditions, our findings suggest that high resource inputs are required to maintain good immune function. It is possible that the high rate of energy

expenditure associated with maternal testosterone exposure combined with the low rates of energy inputs due to poor food conditions left insufficient resources for maintaining the immune system. We found no effect of testosterone treatment on immune function in the chicks reared under poor food conditions but this may be because we performed the immune challenge during the 30 to 37 days after hatching and most chick mortality occurred before this test. As a consequence we lacked sufficient statistical power to properly test this, plus the fledglings that received the immune challenge were all survivors of the critical period of high mortality earlier in development and therefore represented a biased sample containing better quality chicks. Therefore, we cannot discount the possibility that the high number of testosterone chicks that perished under poor food conditions may have died because they had very weak immune systems.

The context-dependent effects of yolk androgens we proposed may explain the relatively frequent inconsistencies in the effects coming from maternal androgen manipulation studies in bird species. As listed in table 4.1 of von Engelhardt and Groothuis (2011), 7 studies showed an increase in growth, 2 a decrease, and 8 other studies showed no or inconsistent results. Likewise, 4 studies showed an increase in survival, 3 a decrease and 3 no effect. Interestingly, the most cited study for the detrimental effects of yolk testosterone on survival, the study on American kestrels (Sockman and Schwabl 2000), was conducted in a poor food year (Sockman oral comm.). A replication of that study on the closely related Eurasian kestrel in a good year found indications for a positive effect (Dijkstra, Boonekamp and Groothuis, in prep). It is therefore important that in the future such studies should take the food condition into account.

We applied the food treatment only after egg production and incubation. However, avian mothers are also capable of depositing different patterns of testosterone in their clutches under different environments. For example, in domesticated canaries (*Serinus canaria*), the increasing pattern of yolk T was steeper when females were in a high-quality food condition than when females were in a low-quality food condition (Vergauwen *et al.* 2012). In zebra finches (*Taeniopygia guttata*), the concentration of yolk androgens decrease over the laying sequence (Gil *et al.* 1999; Griffith *et al.* 2011) and Sandell *et al.* (2007) found that this decreasing pattern was present when provided with a low quality diet, but flattened when given a high quality diet. Although these two species produce different patterns of within-clutch variation in yolk androgens, these results suggest that females have flexibility in the patterns of yolk androgen deposition and can potentially adjust them according to their predictions about food availability during the chick-rearing season, which we are currently investigating in the same population of feral pigeons.

In conclusion, our results indicate the co-evolution of two pathways for maternal effects that are adaptive for the mother and, because of her favouritism for certain offspring, suggest that mothers have the upper hand in the parent-offspring conflict. The context dependent effects should be taken into considerations in future studies when testing potential fitness effects of exposure to elevated levels of maternal testosterone.

Table 1. Final model of non-linear mixed-effects analysis of growth in body mass. For explanations see text.

Variable: Body mass					
Level 1:	Fixed effect: Asym*exp(-b2*b3^Age)				
	Random effect: Chick identity				
Level 2:	Fixed effect: Asym ~ Sex, b2 ~ Egg mass, b3 ~ T injection				
	Random effect: Asym ~ 1, b3 ~ 1				
Factor	Estimate	SE	DF	t	p
Asym.(Intercept)	275.293	4.330	215	63.577	<0.001
Asym.Sex	16.305	6.860	215	2.377	0.018
b2.(Intercept)	5.173	0.332	215	15.561	<0.001
b2.Egg mass	-0.108	0.019	215	-5.712	<0.001
b3.(Intercept)	0.859	0.005	215	183.075	<0.001
b3.T injection	-0.013	0.006	215	-1.950	0.053

Table 2. Final model of non-linear mixed-effects analysis of growth in head bill length. For explanations see text.

Variable: Head-bill length					
Level 1:	Fixed effect: Asym/(1-exp((xmid-Age)/scal))				
	Random effect: Chick identity				
Level 2:	Fixed effect: Asym ~ Sex, xmid ~ Egg mass, scal ~ Egg mass				
	Random effect: Asym ~ 1, xmid ~ 1, scal ~ 1				
Factor	Estimate	SE	DF	t	p
Asym.(Intercept)	45.117	0.303	199	149.016	<0.001
Asym.Sex	0.737	0.463	199	1.591	0.113
xmid.(Intercept)	2.063	1.168	199	1.767	0.079
xmid.Egg mass	-0.136	0.067	199	-2.026	0.044
scal.(Intercept)	9.052	1.408	199	6.429	0.000
scal.Egg mass	-0.216	0.080	199	-2.690	0.008

Table 3. Final model of non-linear mixed-effects analysis of growth in tarsus length. For explanations see text.

Variable: Tarsus length					
Level 1:	Fixed effect: Asym/(1-exp((xmid-Age)/scal))				
	Random effect: Chick identity				
Level 2:	Fixed effect: Asym ~ Sex + Egg mass, xmid ~ Egg mass, scal ~ 1				
	Random effect: Asym ~ 1, xmid ~ 1, scal ~ 1				
Factor	Estimate	SE	DF	t	p
Asym.(Intercept)	30.932	2.203	199	14.042	<0.001
Asym.sex	0.962	0.343	199	2.805	0.006
Asym.Egg mass	0.311	0.127	199	2.445	0.015
xmid.(Intercept)	6.442	1.099	199	5.862	<0.001
xmid.Egg mass	-0.169	0.063	199	-2.683	0.008
scal.(Intercept)	4.461	0.087	199	51.486	<0.001

Table 4. Final model of non-linear mixed-effects analysis of growth in wing length. For explanations see text.

Variable: Wing length					
Level 1:	Fixed effect: Asym/(1-exp((xmid-Age)/scal))				
	Random effect: Chick identity				
Level 2:	Fixed effect: Asym ~ Sex, xmid ~ Egg mass, scal ~ 1				
	Random effect: Asym ~ 1, xmid ~ 1				
Factor	Estimate	SE	DF	t	p
Asym.(Intercept)	185.848	1.722	216	107.912	<0.001
Asym.sex	4.467	2.100	216	2.127	0.035
xmid.(Intercept)	19.463	1.436	216	13.554	<0.001
xmid.egg mass	-0.289	0.082	216	-3.532	0.001
scal.(Intercept)	5.133	0.052	216	99.075	<0.001

Appendix A. All candidate models (fixed effects at level 2) for body mass growth curve analysis

Variable: Body mass				
Asym	b2	b3	AIC	Loglikelihood
Asym~1	b2~1	b3~1	1774.92	-880.46
Asym~Sex	*b2~1*	*b3~1*	*1771.68*	*-877.84*
Asym~T	b2~1	b3~1	1776.21	-880.11
Asym~T*Sex	b2~1	b3~1	1774.03	-877.01
Asym~Eggmass	b2~1	b3~1	1776.57	-880.28
Asym~Sex+Eggmass	b2~1	b3~1	1773.68	-877.84
Asym~Tinj+Eggmass	b2~1	b3~1	1777.63	-879.82
Asym~1	b2~Sex	b3~1	1776.49	-880.24
Asym~1	b2~T	b3~1	1775.02	-879.51
Asym~1	b2~T*Sex	b3~1	1778.62	-879.31
Asym~1	b2~Eggmass	b3~1	1767.30	-875.65
Asym~1	b2~Sex+Eggmass	b3~1	1768.45	-875.22
Asym~1	*b2~T+Eggmass*	*b3~1*	*1767.21*	*-874.61*
Asym~1	b2~1	b3~Sex	1776.92	-880.46
Asym~1	b2~1	b3~T	1774.63	-879.32
Asym~1	b2~1	b3~T*Sex	1778.60	-879.30
Asym~1	b2~1	b3~Eggmass	1771.22	-877.61
Asym~1	b2~1	b3~Sex+Eggmass	1773.13	-877.56
Asym~1	*b2~1*	*b3~T+Eggmass*	*1770.11*	*-876.05*
Asym~Sex	b2~T+Eggmass	b3~T+Eggmass	1765.59	-870.80
Asym~1	b2~T+Eggmass	b3~T+Eggmass	1768.33	-873.17
Asym~Sex	b2~T	b3~T+Eggmass	1768.69	-873.34
Asym~Sex	b2~Eggmass	b3~T+Eggmass	1764.10	-871.05
Asym~Sex	b2~1	b3~T+Eggmass	1767.28	-873.64
Asym~1	b2~Eggmass	b3~T+Eggmass	1766.91	-873.45
Asym~Sex	**b2~Eggmass**	**b3~T**	**1763.43**	**-871.71**
Asym~Sex	b2~Eggmass	b3~Eggmass	1765.04	-872.52
Asym~Sex	b2~Eggmass	b3~1	1764.18	-873.09

T = T treatment, T*Sex= T treatment + Sex+ T treatment × Sex

The order of the models followed our strategy of model selection. The models in italics are the best models for each parameter and the final model is bold.

Appendix B. All candidate models (fixed effects at level 2) for head-bill length growth curve analysis

Variable: Head-bill length				
Asym	xmid	scal	AIC	Loglikelihood
Asym~1	xmid~1	scal~1	544.55	-262.27
Asym~Sex	*xmid~1*	*scal~1*	*544.27*	*-261.14*
Asym~T	xmid~1	scal~1	546.00	-262.00
Asym~T inj.*Sex	xmid~1	scal~1	547.07	-260.53
Asym~Eggmass	xmid~1	scal~1	544.85	-261.43
Asym~Sex+Eggmass	xmid~1	scal~1	544.91	-260.45
Asym~Tinj+Eggmass	xmid~1	scal~1	545.82	-260.91
Asym~1	xmid~Sex	scal~1	545.89	-261.94
Asym~1	xmid~T	scal~1	546.43	-262.21
Asym~1	xmid~T*Sex	scal~1	549.65	-261.82
Asym~1	*xmid~Eggmass*	*scal~1*	*545.43*	*-261.71*
Asym~1	xmid~Sex+Eggmass	scal~1	546.62	-261.31
Asym~1	xmid~T+Eggmass	scal~1	547.18	-261.59
Asym~1	xmid~1	scal~Sex	545.16	-261.58
Asym~1	xmid~1	scal~T	545.66	-261.83
Asym~1	xmid~1	scal~T*Sex	547.97	-260.99
Asym~1	*xmid~1*	*scal~Eggmass*	*543.52*	*-260.76*
Asym~1	xmid~1	scal~Sex+Eggmass	544.59	-260.29
Asym~1	xmid~1	scal~T+Eggmass	544.12	-260.06
Asym~Sex	**xmid~Eggmass**	**scal~Eggmass**	**541.18**	**-257.59**
Asym~Sex	xmid~Eggmass	scal~1	545.43	-260.71
Asym~1	xmid~Eggmass	scal~Eggmass	541.45	-258.72
Asym~Sex	xmid~1	scal~Eggmass	542.93	-259.46

T = T treatment, T*Sex= T treatment + Sex+ T treatment × Sex

The order of the models followed our strategy of model selection. The models in italics are the best models for each parameter and the final model is bold.

Appendix C. All candidate models (fixed effects at level 2) for tarsus length growth curve analysis

Variable: Tarsus length				
Asym	xmid	scal	AIC	Loglikelihood
Asym~1	xmid~1	scal~1	456.46	-218.23
Asym~Sex	xmid~1	scal~1	450.92	-214.46
Asym~T	xmid~1	scal~1	458.20	-218.10
Asym~T inj.*Sex	xmid~1	scal~1	454.61	-214.30
Asym~Eggmass	xmid~1	scal~1	453.48	-215.74
Asym~Sex+Eggmass	*xmid~1*	*scal~1*	*448.96*	*-212.48*
Asym~Tinj+Eggmass	xmid~1	scal~1	454.30	-215.15
Asym~1	xmid~Sex	scal~1	458.21	-218.11
Asym~1	xmid~T	scal~1	458.39	-218.20
Asym~1	xmid~T*Sex	scal~1	462.02	-218.01
Asym~1	*xmid~Eggmass*	*scal~1*	*453.56*	*-215.78*
Asym~1	xmid~Sex+Eggmass	scal~1	455.47	-215.73
Asym~1	xmid~T+Eggmass	scal~1	454.89	-215.45
Asym~1	xmid~1	scal~Sex	458.46	-218.23
Asym~1	xmid~1	scal~T	456.54	-217.27
Asym~1	xmid~1	scal~T*Sex	460.38	-217.19
Asym~1	xmid~1	scal~Eggmass	458.33	-218.21
Asym~1	xmid~1	scal~Sex+Eggmass	460.43	-218.21
Asym~1	xmid~1	scal~T+Eggmass	458.24	-217.12
Asym~Sex+Eggmass	**xmid~Eggmass**	**scal~1**	**446.96**	**-210.48**
Asym~Sex	xmid~Eggmass	scal~1	448.69	-212.34

T = T treatment, T*Sex= T treatment + Sex+ T treatment × Sex

The order of the models followed our strategy of model selection. The models in italics are the best models for each parameter and the final model is bold.

Appendix D. All candidate models (fixed effects at level 2) for wing length growth curve analysis

Variable: Wing length				
Asym	xmid	scal	AIC	Loglikelihood
Asym~1	xmid~1	scal~1	1291.88	-637.94
Asym~Sex	*xmid~1*	*scal~1*	*1290.37*	*-636.19*
Asym~T	xmid~1	scal~1	1292.84	-637.42
Asym~T inj.*Sex	xmid~1	scal~1	1292.40	-635.20
Asym~Eggmass	xmid~1	scal~1	1293.82	-637.91
Asym~Sex+Eggmass	xmid~1	scal~1	1291.83	-635.91
Asym~Tinj+Eggmass	xmid~1	scal~1	1294.84	-637.42
Asym~1	xmid~Sex	scal~1	1293.32	-637.66
Asym~1	xmid~T	scal~1	1292.84	-637.42
Asym~1	xmid~T*Sex	scal~1	1295.05	-636.52
Asym~1	xmid~Eggmass	scal~1	1284.60	-633.30
Asym~1	xmid~Sex+Eggmass	scal~1	1284.71	-632.35
Asym~1	*xmid~T+Eggmass*	*scal~1*	*1284.41*	*-632.20*
Asym~1	xmid~1	scal~Sex	1292.98	-637.49
Asym~1	xmid~1	scal~T	1293.46	-637.73
Asym~1	xmid~1	scal~T*Sex	1295.14	-636.57
Asym~1	xmid~1	scal~Eggmass	1293.73	-637.86
Asym~1	xmid~1	scal~Sex+Eggmass	1294.56	-637.28
Asym~1	xmid~1	scal~T+Eggmass	1295.37	-637.68
Asym~Sex	xmid~T+Eggmass	Scal~1	1282.85	-629.42
Asym~Sex	**xmid~Eggmass**	**scal~1**	**1282.30**	**-631.15**

T = T treatment, T*Sex= T treatment + Sex+ T treatment × Sex
The order of the models followed our strategy of model selection. The models in italics are the best models for each parameter and the final model is bold.

Appendix E. Macronutrient components of grain mixtures in food treatment.

Name of grain mixture	Tortelduivenvoer (KASPER™ 6721)	Sierduivenvoer (KASPER™ 6712)	Duivenkorrel[a] (KASPER™ P40)	Gemengd graan met broken mais
Protein	11.90%	13.30%	15.60%	10.10%
Fat	5.20%	3.40%	2.80%	2.40%
Cellulose	6%	4.40%	2.10%	2.80%
Ash	2.40%	2.40%	4%	1.60%
Standard food	*ad lib.*	*ad lib.*		
Good food[b]	*ad lib.*	*ad lib.*	*ad lib.*	
Poor food				33g /pair-day

[a] also contains calcium 0.6%, potassium 0.6%, lysine 6g, sodium 0.1%, E 672 vitamin A 19000 lE/kg, vitamin E 100 lE/kg, E 671 vitamin D3 300 lE/kg, and copper 12mg/kg

[b] also supplemented with Supralith ™ supplementation powder every one or two days.

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Dutch summary - Nederlandse samenvatting

Introductie

In planten en dieren geven moeders hun genen door aan hun nakomelingen. Zij kunnen hun nakomelingen echter ook beïnvloeden op een niet-genetische wijze, door de omgeving waarin de nakomelingen zich, te veranderen. Moeders kunnen bijvoorbeeld grotere eieren of zaden produceren, waardoor de jongen groter worden. Moeders kunnen ook de hoeveelheid van verschillende voedingstoffen die zij aan hun nakomelingen geven variëren, wat de kwaliteit van de nakomelingen beïnvloedt.
Moeders kunnen ook het tijdstip bepalen waarop een nakomeling zijn embryonale ontwikkeling begint in relatie tot andere nakomelingen (en dus ook wanneer zij uit het zaadje, ei of de baarmoeder komen), wat zowel na- als voordelen kan hebben voor de nakomelingen in competitie met andere nakomelingen. Het overbrengen van moederlijke hormonen aan de nakomelingen is een extra manier voor moeders om het ontwikkelingsproces van hun nakomelingen te beïnvloeden. Hormonen zijn signalen die de fysiologie en het gedrag in planten en dieren regelen, en spelen een belangrijke rol in het coördineren van ontwikkeling. Daarom kunnen hormonen die moeders overdragen naar zaden, eieren of direct naar het embryo in de baarmoeder de manier waarop hun nakomelingen ontwikkelen, sterk beïnvloeden.
Androgenen, zoals testosteron, zijn hormonen die veel aspecten van voortplantingsfysiologie en gedrag beïnvloeden bij volwassenen en staan al lang bekend om betrokkenheid bij veel aspecten van de ontwikkeling van gewervelde dieren, zoals de seksuele differentiatie. Twintig jaar geleden werd gevonden dat vogeleieren hoge concentraties androgene hormonen bevatten die systematisch in concentratie varieerden binnen nesten, tussen nesten van dezelfde moeder en ook tussen soorten. In die tijd hadden vogels al bewezen zeer goede diermodellen te zijn op het gebied van de gedragsecologie voor het onderzoeken van veel verschillende typen van moederlijke reproductieve investeringen, omdat alle moederlijke stoffen overgebracht worden naar de nakomelingen in een groot verzegeld ei, die zich ontwikkelt buiten het lichaam van de moeder. Hierdoor kan er gemakkelijk gemeten en gemanipuleerd worden met verschillende aspecten van de samenstelling van het ei ,en het begin of de temperatuur van de broedperiode.

Sindsdien heeft uitgebreid experimenteel bewijs zich opgestapeld voor diverse effecten van verhoging van de concentratie van moederlijke testosteron in de eieren op de ontwikkelingen van nakomelingen tijdens de prenatale periode, de postnatale periode en zelfs tijdens de adolescentie en volwassenheid. Deze studies manipuleerde de concentratie van het testosteron in de eierdooiers meestal door de dooier in de eieren in te spuiten met testosteron opgelost in olie. De dooier biedt de embryo's continue voedingstoffen en andere moederlijke stoffen (o.a. testosteron) tijdens de gehele prenatale ontwikkeling. Prenatale blootstelling aan dooiers met meer testosteron zorgt ervoor dat kuikens competitiever zijn door snellere groei, verhoogd bedelgedrag voor ouderlijk voedsel, agressiever gedrag dat kan resulteren in een betere overlevingskans, maar dit gaat soms ten koste van het metabolisme en het immuunsysteem. Testosteron in de eieren blijkt niet alleen systematisch te variëren tussen opeenvolgende eieren binnen een nest in verschillende patronen, maar ook tussen nesten overeenkomstig variatie in de omgeving, zoals beschikbaarheid aan voedsel, kwaliteit van het mannetje of de conditie van de moeder. Het blijkt ook te varieren tussen soorten in relatie tot hun ecologische aanpassingen. Dit inspireerde verschillende interessante hypotheses, zowel over de evolutionaire oorsprong van de opgemerkte patronen alls over de fysiologische mechanismen die ten grondslag liggen aan deze patronen.

Dit proefschrift evalueert verschillende van deze evolutionaire en mechanistische hypothesen. Ik heb deze hypotheses getest door beschrijvende en experimentele studies aan individuen van dezelfde soort, en door het onderzoeken van de mogelijke evolutionaire verklaringen van soortsvariatie in moederlijke hormoonoverdracht door die variatie te correleren met andere aspecten van hun soortspecifieke biologie.

Effecten moederlijke hormoonoverdracht: Selectie op de moeder of op het ei?

Als eerste heb ik een fundamenteel onbeantwoorde aspect onderzocht, namenlijk de vraag of moederlijke testosteron in eidooiers (dat de ontwikkeling van de nakomelingen beïnvloedt) correleert met testosteron in de bloedsomloop van de moeder (dat het gedrag en fysiologie van de moeder beïnvloedt). Dit heeft belangrijk implicaties voor de mogelijkheid om onafhankelijk van elkaar zich tijdens de evolutie te ontwikkelen en aan te passen.
Omdat het meeste moederlijke testosteron dat geproduceerd wordt tijdens het leggen van de eieren van het folliculaire weefsel rond de groeiende dooiers in de eierstokken komt, is het aannemelijk dat soorten met een hoge testosteron productie, hogere testosteron concentraties in zowel de dooiers als de bloedsomloop van de moeder hebben. Dit resulteert in een positieve correlatie tussen testosteron in dooiers en testosteron in de plasma van de moeder. En inderdaad, in mijn soortvergelijkende studie beschreven in **Hoofdstuk 2,** vond ik een dat soorten met een hoge concentraties van testosteron in plasma van de moeder ook een hoge concentratie van testosteron in de dooiers lieten zien.

Wanneeer in deze analyse de grootte van het legsel (d.w.z. het aantal eieren in een nest, dus het aantal actieve follikels) werd betrokken, werd de positieve correlatie tussen testosteron in vrouwelijke plasma en testosteron in dooiers nog sterker. Legsel grootte op zichzelf correleert ook direct met testosteron in vrouwelijk plasma, wat aangeeft dat de hoeveelheid moederlijke testosteron dat wordt uitgescheiden naar de bloedsomloop van de moeder, ook afhangt van het aantal hormoon producerende follikels. Deze bevindingen suggereren dat testosteron dat wordt geproduceerd in de eierstokken en terecht komt zowel in de dooiers als in de bloedsomloop van de moeder, niet onafhankelijk gereguleerd worden bij vogels.

De effecten van testosteron circulerend in het bloed van de moeder en de effecten van blootstelling aan moederlijk testosteron tijdens embryonale ontwikkeling zijn zeer verschillend, evenals de ecologisch en omgevingsuitdagingen van moeder en kind. Daarom is het waarschijnlijk dat de druk die uitgeoefend wordt door natuurlijke selectie op testosteron in moederlijke plasma en testosteron in dooiers substantieel verschillen in zowel richting als intensiteit. In **Hoofdstuk 2** testte ik of testosteron in de moeder en in de dooier correleren met dezelfde ecologische variabelen en dus of beiden onder identieke selectiedruk staan of niet. Ik vond dat over evolutionaire tijd, de productie van moederlijke testosteron zich heeft aangepast aan functies die belangrijk zijn voor de nakomelingen. Ik vond associaties tussen koloniaal broeden (in tegenstelling tot solitair broeden in grote gebieden voor alle doeleinden) en de incubatie periode (het aantal dagen tussen het leggen van de eieren en het uitkomen van de eieren) met testosteron in dooiers, maar niet met testosteron in moederlijke plasma. De gegevens suggereren dat evolutie een hoger testosteron niveau in de eierstokken heeft produceerd in koloniaal broedende vogelsoorten en een lagere produktie in soorten met een relatieve korte incubatieperiode, beiden ten gunste van de kuikens. Korte broedperiodes waren ook geassocieerd met hoge testosteron concentraties in mannelijk plasma, maar niet in vrouwelijk plasma. Ik interpreteerde het effect in mannen als een gevolg van een correlatie met een derde variabele, namenlijk een hoge predatie druk, aangezien aangetoond is dat een hoge predatie druk een sterke selectieve druk is voor het verkorten van de incubatie periode bij van verschillende vogelsoorten. Ik suggereer dat als het predatierisico hoog is (wat voor lage incubatieperiodes zorgt) mannen voordeel hebben van hoge testosteron concentraties, omdat dit ervoor zorgt dat zij langere periodes op en van het nest af zijn en de vermindering van trips van en naar het nest de kans van het aantrekken van roofdieren vermindert. Anderzijds, blootstelling aan testosteron in dooiers vergroot opvallend vertoon van bedelen door kuikens, en dit zou juist roofdieren tot het nest aantrekken. Dit verklaard waarom dooiers lijken te zijn geselecteerd om een lage testosteron concentratie te bevatten in roofdier-rijke omgevingen.
Het feit dat predatiedruk wel effect heeft op testosteron spiegels in vaders maar niet in moeders zou een gevolg kunnen zijn van de beperking die selectie kan uitoefenen op moederlijke testosteron gegeven de selectiedruk op testosteron in dooiers omdat deze aan elkaar gekoppeld zijn (zie boven).

Effecten moederlijke hormoonoverdracht in relatie tot competitie tussen nestgenootjes

Veel vogelsoorten leggen meerdere eieren in een nest, meestal met een dag ofmeer tussenpoze. In veel vogelsoorten neemt de concentratie van testosteron toe over de legvolgorde. Een belangrijke hypothese hiervoor is dat moeders hierdoor compenseren voor het feit dat de later gelegde eieren later uitkomen en dat de kuiken daardoor een nadeel hebben in de competitie met oudere nestgenoten. De vraag waarom moeders er voor zorgen dat de eieren asynchroon uitkomen door al te gaan broeden op de eerst gelegde eieren voordat de laatste eieren gelegd zijn, en vervolgens deze effecten tegenwerken via verhoogde testosteron toewijzing aan de laatst gelegde eieren is al jaren een onbeantwoorde vraag. Een oplossing is dat de effecten van de differentiele toewijzing van testosteron afhangt van de omgevingscontext en samenwerken met context afhankelijke effecten van asynchroon uitbroeden. Asynchroon uitbroeden faciliteert dat het aantal kuikens snel vermindert ten tijde van weinig voedsel via uithongering van laat uitgekomen kuikens die niet kunnen competeren met oudere broertjes en zusjes, maar staat overleving toe als er voedsel in overvloed is. Echter, in dat laatste geval hebben de laat uitgekomen kuikens die nu moeten overleven een nadeel. Een toenemend patroon van moederlijke testosteron in de volgorde van de gelegde eieren kan dan hulp bieden aan laat uitgekomen kuikens door te compenseren van hun competitieve nadelen en hun overlevingskans verhogen. De negatieve effecten van laat uitgekomen kuikens in slechte jaren worden verergerd door het extra ondermijnen van de overleving van laat uitgekomen kuikens doordat moederlijk testosteron ook negatieve effecten kan hebben zoals op het metabolisme en het immuunsysteem, die de kuikens niet het hoofd kunnen bieden wanneer er niet voldoende voedsel is. Tot nu toe zijn zulke context afhankelijke effecten van moederlijke testosteron nog niet direct getest.

Om deze hypothese te testen hebben wij een experiment uitgevoerd in rotsduiven, zoals beschreven in **Hoofdstuk 6.** Rotsduiven produceren nesten met twee eieren, waarin de concentraties van moederlijke testosteron in de dooiers aanzienlijk hoger is in het tweede ei dan in het eerste ei. In een experiment waarin wij zowel de concentraties testosteron in de eieren en als de voedsel omstandigheden manipuleerden, vonden wij dat onder goede voedselomstandigheden de kuikens van testosteron geïnjecteerde eieren (T-kuikens) sneller groeiden dan hun pleegbroertjes en zusjes uit de controle groep. Onder beperkte voedsel omstandigheden hadden T-kuikens een lagere overleving dan de controle kuikens maar niet onder goede voedselomstandigheden. De verklaring kan zijn dat embryonale blootstelling aan testosteron een verhoging van het energetische kostbare competitieve gedrag veroorzaakt, wat een voordeel zou zijn als voedsel in overvloed aanwezig is (omdat dit resulteert in een snellere groei van T-kuikens dan in de controle kuikens, **Hoofdstuk 6**), maar in een schadelijke, negatieve energie balans resulteert als voedsel ontoereikend is voor deze verhoogde energie uitgaven. Onze bevindingen ondersteunen onze hypothese dat een patroon van toenemende hoeveelheid testosteron over de gelegde volgorde van de eieren een moederlijke strategie kan ondersteunen, waarin alle kuikens overleven in goede jaren, maar later uitgekomen kuikens overlijden in slechte jaren waarin voedsel onvoldoende aanwezig is om alle jongen groot te brengen.

Wat onopgelost blijft is waarom soorten zoals duiven over de legvolgorde een consequent toenemend patroon van testosteron in dooiers produceren, terwijl andere soorten een vlak patroon laten zien en weer andere soorten een daling in testosteron concentraties over de legvolgorde. Tegelijkertijd zijn er veel soorten die nesten produceren met slechts kleine verschillen in grootte tussen nestgenootjes (via geringe asynchrony in het uitbroeden van de eieren of een relatieve langzame groei van eerst uitgekomen kuikens), terwijl andere soorten nesten produceren met juist een groot verschil in grootte tussen nestgenootjes (via sterke asynchrony of snelle kuikengroei). Hoe groter het berschil in grootte is tussen de eerste en de laatste uitgekomen kuikens, hoe moeilijkere het is voor de laatst uitgekomen kuikens om

effectief te concurreren om voedsel van de ouders. Ik stelde de volgende hypothese voor: 1) zou het kunnen zijn dat in sommige soorten, zoals de duif, moeders een toenemend patroon van testosteron concentraties produceren om zich zo te richten op de overleving van het hele nest in het geval dat de voedsel omstandigheden goed zijn, en dit daarom combineren met een kleiner verschil in lichaamsgrootte tussen haar kinderen zodat de laatst uitgekomen kuikens ook werkelijk in staat zijn om hun competitieve nadelen te overkomen in goede jaren jaren? En omgekeerd, 2) zou het kunnen zijn dat andere soorten een vlak of verminderd patroon produceren, gecombineerd met een groter verschil in grootte tussen de kinderen met het doel om de overbodige als laatste uitgekomen kuikens (die alleen maar geproduceerd worden als verzekering voor het geval dat de eerste eieren niet uitkomen) te elimineren, zodat zij juist geen compensatie door middel van testosteron moeten hebben? In **Hoofdstuk 3** beschrijf ik mijn analyses, waarbij ik waarden voor een gevarieerde set van vogelsoorten combineer, waarin ik vond dat soorten die een toenemend patroon van testosteron in dooiers produceren, ook een klein verschil in lichaamsgrootte van de kinderen produceren terwijl soorten met een omgekeerd patroon juist een groot verschil in kuikengrootte produceren. Dit ondersteunt onze hypothese en verklaart de grote variatie tussen soorten in patronen van testosteron in dooiers over de gelegde volgorde.

Echter,in dit verband bestaat er nog een andere onopgeloste vraag: een afnamen van testosteron concentraties in de dooier over de legvolgorde is geinterpreteerd als een slim mechanisme om juist nest reductie te ondersteunen in zogenoemde siblicidal soorten – waar eerst uitgebroede kuikens de jongere kuikens vermoorden, vooral als zij bedreigd worden met de hongerdood. Zo'n afname in testosterone over de legvolgorde is gerapporteerd voor de siblicidal koereiger, en dit werd geïnterpreteerd as een moederlijke strategie om de sociale dominante van het eerst uitgekomen kuiken te ondersteunen. Maar sindsdien hebben verschillende studies lakke of toenemende testosteron patronen in eieren van siblicidal soorten gerapporteerd. In **Hoofdstuk 4** laat ik zien dat in de siblicidal drieteenmeeuw de concentraties testosteron en androsteendion (een ander androgeen van waaruit testosteron gemaakt kan worden) veel groter zijn in het tweede gelegde ei dan in het eerste gelegde ei, tegenovergesteld dus aan het patroon in de koereiger. Dit roept twijfel op over het idee dat blootstelling aan testosteron in dooiers doeltreffend siblicide zou ondersteunen. Vervolgens voerde ik een experiment uit om te testen of verhoogde blootstelling aan moederlijke androgenen zorgde voor toenemend agressie en bedel gedrag naar de ouders om voedseloverdracht te stimuleren. Tot mijn verbazing bleek dat blootstelling aan verhoogde spiegels van androgenen, zoals voorkomt bij laatst uitgekomen kuikens, zorgde voor toenemend agressie naar nestgenootjes, en een hogere sociale dominantie. Dat is vreemd omdat deze potentie voor agressie in de laatst gelegde eieren duidelijk onderdrukt is in een natuurlijke omgeving, aangezien kuikens van tweede eieren (met dus een verhoogde concentratie van testosteron) juist subdominant zijn vanwege de substantiële kleinere lichaamsomvang. Dit creëert de volgende paradox: nestgenoten van siblicidal soorten zoals de drieteenmeeuw, creëren sterkte dominante hiërarchieën, waarin de eerste uitgebroede kuikens altijd de dominante status heeft en duwt, pikt en soms zelfs zijn concurrerende, minderwaardige, jongere broer/zus, dat feitelijk komt uit een ei met een hogere testosteron concentratie, vermoord. Daarbij is de jongere nestgenoot nooit agressief en vecht niet terug. Als de verhoogde blootstelling aan testosteron in kuikens van het 2^{e} ei nooit hun aggressie verhoogd, vanwege hun subdominante positie, (ze zouden toch ook nooit winnen van de groitere broer of zus), waarom stoppen moeders dan meer androgenen in die eieren?

Drieteenmeeuwen broeden in dichte kolonies op richels van steile rotsen en de eerst uitgekomen kuikens elimineren hun nestgenoot via een woeste pik-aanval, wat uiteindelijk het jongere kuiken uit het nest drijft. Vaak vallen deze uitgeworpen kuikens in een ander nest op een lagere richel en in 8% van zulke gevallen worden zij geadopteerd door nieuwe families, doordat ouders niet hun jongen herkennen totdat zij een paar weken oud zijn. Als verdreven kuikens in nesten vallen die andere kuikens van dezelfde of kleinere grootte bevatten, dan zou prenatale blootstelling aan verhoogde moederlijke androgenen hen helpen een vreemd nest binnen te dringen en een dominante status te verkrijgen, waardoor zij toch overleven. De ''adoptie faciliterings hypothese'' is in overeenstemming met eerdere bevindingen dat moeders neigen

om de concentratie van androgenen in dooiers te verhogen onder slechte voedsel condities (wanneer er meer siblicidal gedrag is) en tijdens een hogere dichtheid van nesten (**Hoofdstuk 2)** in soorten die adoption laten zien. Dit zou het succes van verdreven kuikens.

Testosteron is al veel eerder geïdentificeerd als een krachtig middel voor het verhogen van agressie, niet via prenatale invloeden van moederlijke testosteron op het ontwikkelen van agressief gedrag, maar via de eigen produktie van testosteron in volwassen dieren. Sindsdien is ook aangetoond dat testosteron territoriale agressie stimuleert in zeer jonge kokmeeuwen. Het speelde echter geen rol in de agressie tussen nestgenoten bij deze niet-siblicidal soort. Of testosteron een rol speelt in de extreem heftige aanvallen die voorkomen tussen nestgenoten in siblicial soorten was nog nooit onderzocht, ondanks jarenlange speculaties hioerover in de literatuur. In een veldexperiment testte ik, voor de eerste keer, of testosteron ervoor zorgt dat siblicidal agressie toeneemt in kuikens van drieteenmeeuwen (**Hoofdstuk 5**). Ik implanteerde kuikens met of een testosteron afgevende capsule of met een lege capsule (controle groep) en bracht testosteron kuikens en controle kuikens van dezelfde leeftijd en grootte samen in een nest. Ik vond dat vergeleken met de controle kuikens, de testosteron kuikens agressiever werden tegenover hun metgezel. Zij werden ook agressiever tegen een gesimuleerd roofdier en zij bedelden ook meer naar de (pleeg)ouders voor voer. Dit geeft aan dat circulerend testosteron een krachtig middel is voor het verbeteren van het competitieve vermogen. Dit is het eerste experimentele bewijs dat testosteron vroeg na de geboorte agressie verhoogd in een context van siblicide. Ik vond ook dat de grotere kuikens in de experimentele paren agressievere kuikens waren, hetgeen consistent is met het feit dat de oudere, grotere nestgenoten in drieteenmeeuwen sociaal dominanter zijn en de meeste agressie vertonen. Daarnaast waren de kuikens met een laag lichaamsgewicht agressiever dan de kuikens met een hoog lichaamsgewicht, wat het idee ondersteunt dat door honger agressie in siblicidal soorten toeneemt.

Conclusie

Dit proefschrift onderzoekt verschillende belangrijke hypotheses over de functie en evolutie van de variatie in moederlijke hormoonoverdracht en integreert vragen over de mechanismen met vragen over evolutionaire processen. Ik vond bewijs dat moederlijke testosteron in eierdooiers is geëvolueerd om het functioneren van de nakomelingen te bevorderen, ook al correleren hormoonwaardes in de eieren met die van de moeder. Dit laatste kan een compromis weerspiegelen tussen selectie op de kuikens en selectie op de moeder. Ik toon aan dat patronen van dooier testosteron over de legvolgorde correleert met de verschillen in grootte tussen nestgenootjes en dat dit moederlijke strategieën ondersteunt voor de overleving van alle nestgenootjes versus het elimineren van laat uitgebroede kuikens, afhankelijk van en aangepast aan de voedselcontext. Ik toon aan dat in en soort met een toenemend patroon van testosteron in dooiers over de legvolgorde, de effecten van testosteron in dooiers voordelig kunnen zijn voor de laatst uitgekomen en relatief zwakke kuikens als voedsel in overvloed is en schadelijk als voedsel schaars is, hetgeen de kans op optimaal broedsucces voor de moeder verhoogd. Ik toon ook aan dat in andere soorten waar oudere kuikens de jongere kuikens uit het nest gooien toch een toenemend patroon van testosteron over de legvolgorde bestaat, dat deze verhoogde hormoonwaardes voor toenemende agressie tussen nestgenootjes zorgt, hetgeen een rol zou kunnen spelen in het faciliteren van adoptie in andere nesten. Tenslotte toon ik voor de eerste keer aan dat in soorten waar oudere kuikens agressief zijn tegen jongere kuikens, testosteron in de bloedsomloop deze agressie naar nestgenoten verhoogd.

Acknowledgements

Thank you Ton for answering an out-of-the-blue email from a student in the States eager to work with you, for hosting me as a Fulbright fellow, and later for giving me this PhD position. Thank you for having so much faith in me to give me the freedom to pursue questions that I was curious about. I could always count on you to be open to discussing even my craziest thoughts and you taught me how to consider every possible angle of a idea. I have learned a great deal during these years in your group and have had many unforgettable and instructive experiences.

Cor, it was always enjoyable to work with you and the pigeons. Thanks for always being interested, helpful and making time for me when I needed it. Thanks Simon Verhulst, I learned a lot from your constructively critical feedback over the years. Jean-Cristophe – how lucky was I when a fellow Swiss-with-a-North-American-mentality joined the group? It was always great to talk to someone who gets where I'm coming from!

Gerard, I could always count on you for anything, from helping me shop for field supplies the night before an emergency trip to the arctic, to teaching me to use power tools and wire electrical lamps in my experiment rooms, to putting my phone contract under your name. Thanks!

To the animal caretakers, Sjoerd, Roelie, Saskia and others - thanks for showing me how things work in the animal care facilities, your patience and enthusiasm for teaching me Dutch, and your contributions in taking care of the animals. Roelie, thanks for your help with DNA extractions and PCR. To Vivian, Bonnie and Ilse, thanks for teaching me how to do hormone extractions and assays and for your help in the lab. Julia Groothuis, thanks for agreeing to help translate my Dutch summary at the eleventh hour - I really appreciate it!

To the many postdocs present during my years in Groningen – thanks for your support. Bernd, thanks for the help and animated discussion. Martine, the "other Martina", it was great when you joined our group - I always highly valued our scientific chats. Peter Korsten, the perfect flatmate, thanks for the fun scientific and statistical discussions that helped distill ideas in my head during my last months in Groningen.

To my fellow PhD students and Groningen pals: Marina and Casper, thank you for adopting me into your family, I will forever hide under your porch and you know why. Rie, my Danish, fast-talking, fellow vegan, partner in crime (with shorter legs): it looks like I survived the last year of my PhD without you present but I missed you terribly! Looking forward to future food adventures around the globe (poolparty)! Berber, I am glad I was able to share so many experiences with you during our PhD! Betka and Chris, thanks for all the fun times and knitting/crochet therapy! Giulia thanks for being such a good friend through the most difficult period, I'm so glad you found your happiness in G-town! Vivian thanks for showing me the ropes when I first came to Groningen and for letting me work on your project. I wish you all the best in Germany. Additional PhD students from my cohort: Sara, Kristina, Martijn, Ralf, Kristin, Egbert, Sandra, Mirre, Jelle, Michael, Gert, Els, Nele, Meghan, thanks for your support over the years. Bin-Yan, I wish you the best of luck for the rest of your PhD and hope you can answer many remaining questions about hormone-mediated maternal effects!

To the Master's students who participated in my projects – Yvonne Roelofs, Agnes Waninge, Remco Folkertsma, Peter Kroezen, thank you for the help, it was fun having you on board!

Erpur Snaer Hansen, Roald Hansen, Kjell Einar Erikstad, Borge Moe, Thierry Boulinier – I thank you all for your contributions to my field efforts with the kittiwakes in Iceland and Norway – whether it was arranging permits, teaching me how to rappel down 60 meter cliffs, helping me scoop eggs from nests, or providing food and shelter. I couldn't have done it without you!

Thanks Giacomo Dell'Omo for everything, including being a field guru and mentor during these years in Europe, and for keeping my feet on the ground when my head floated away into abstract concepts and complicated research plans. Thanks also for letting me work on burrow-

nesting seabirds on a warm Mediterranean island to compensate for the arctic cliffs and for giving me your GPS.

Thank you to Dave Anderson for introducing me to behavioral ecology and for turning me into a seabird biologist. Eight months on an island surrounded by little else but boobies kindled my curiosity about the many unusual behaviors and life history strategies of animals and you taught me how to use science to satisfy that curiosity.

Scott McWilliams – my experience with research began with you and I would not be here without you. Thanks for always having faith in me, for being available for help, advice and the occasional tough chat to keep me on track.

Thanks to my friends in North (and South!) America - you all know who you are! Skype, email, Facebook and phones have kept you close even when we were miles apart.

To my wonderful parents and little brothers– no matter where I am on the globe I know I have a warm home to return to and I treasure every moment I spend there. And to my grandmothers, aunts, uncles and cousins in Switzerland– thanks for giving me another loving home in Europe!

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